Distal sensory polyneuropathy in HIV/TB co-infection: the role of vitamin B6 and *N-acetyltransferase 2* genetic variation

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Abstract

Background Both human immunodeficiency virus (HIV) infection and tuberculosis (TB) are complicated by a painful distal sensory polyneuropathy (DSP) that may be due to virus-related HIV-DSP, antiretroviral toxic neuropathy (ATN) or isoniazid-induced peripheral neuropathy (INH-PN). In co-infection with and co-treatment for HIV/TB, DSP risk is increased. Factors driving this risk may be vitamin B6 deficiency and slow metabolism of INH mediated by N-acetyltransferase 2 (NAT2) acetylation, both known risk factors for INH-PN. Supplemental B6 in the form of pyridoxine should prevent INH-PN, but B6 requirements may be increased in HIV/TB because of pre-existing deficiency. The influence of slow acetylation on DSP risk in the context of HIV/TB has not been investigated.

Hypothesis DSP in HIV/TB co-infection is associated with vitamin B6 deficiency and/or slow NAT2 acetylation status.

Method The study design is that of a prospective analytical cohort. Over 1 year, consecutive HIV-infected admissions to a district TB-hospital were assessed at baseline and followed monthly during hospitalisation. Clinical assessments were performed using validated instruments, and DSP was defined by the presence of ≥1 neuropathic symptom and ≥1 neuropathic sign. Vitamin B6 status was determined by plasma pyridoxal 5'-phosphate (PLP) high pressure liquid chromatography. *NAT2* polymerase chain reaction restriction fragment length polymorphism analysis, sequencing and computational haplotyping predicted acetylation phenotype (fast, intermediate or slow). Generalised linear models estimated prevalence ratios (PR)s for baseline risk factors.

Results One hundred and sixteen participants were assessed at baseline on admission. All were receiving standard doses of INH (5 mg/kg/day) as part of a combination anti-TB regimen initiated at the referring facility; 25% were on ART. Prior to admission, only 65% received pyridoxine; on admission 76% were prescribed 25 mg/day and the remainder ≥50 mg/day.

Baseline DSP frequency was 65 (56%). Based on recall of symptom onset, 36% was INH-PN, 22% HIV-DSP and 5% ATN. A further 26% reported concurrent neuropathic and TB symptom onset (prior to anti-TB therapy) and were labelled “TB-DSP”. Previous TB was independently associated with baseline INH-PN (PR 4.2; 95%CI 1.2-14.8). Eighty-one participants had at least one follow-up assessment, and the incidence of DSP in this group was 12.3/100 person-months. Incident DSP was attributed to ATN in 78%.

Twenty-five participants had B6 assessments at baseline and again at either 4 or 8 weeks. At baseline, B6 deficiency (PLP <30 nmol/l) was demonstrated in 1 participant; at follow-up there was no deficiency. Participants receiving higher dose pyridoxine (≥50 mg/day) did not achieve higher levels. PLP levels were no different in those with and without DSP.

NAT2 acetylation phenotype was predicted in a subset of 80 participants: 35% slow, 50% intermediate and 15% fast. Slow acetylation status failed to predict baseline DSP, but a survival analysis revealed a trend for slow acetylators to develop DSP. Slow acetylation did not affect PLP levels.

Conclusion DSP is prevalent in HIV/TB co-infection after the initiation of anti-TB therapy, and DSP continues to develop during concomitant ART. The association of INH-PN with a previous TB history, a previously identified risk factor for ATN, represents a possible priming effect or unmasking of subclinical HIV-DSP. A “TB-DSP” not related to INH-use has been reported anecdotally in the literature and may also represent unmasking of subclinical HIV-DSP. The relationship of DSP to previous and current TB requires further long-term investigation. Pyridoxine supplementation as an adjunct to anti-TB therapy may have prevented the B6 deficiency expected in this cohort, but did not prevent incident DSP cases. However, the contribution of prior deficiency to DSP risk cannot be excluded; pyridoxine supplementation in co-infected patients at the time of anti-TB therapy initiation is recommended. Higher dose pyridoxine (≥50 mg/day) did not equate to higher PLP levels when compared to standard doses of 25 mg/day; in the absence of evidence demonstrating additional neurological benefit, higher dose pyridoxine appears unnecessary. The trend for slow acetylators to develop DSP during anti-TB therapy despite adequate B6 status suggests an additional mechanism for DSP pathogenesis in HIV/TB, or an independent association. Oxidative stress may be driving this process.

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Abbreviations and terminology

4PA	4-pyridoxic acid
acCoA	acetyl co-enzyme A
ADSP	asymptomatic distal sensory polyneuropathy
ALT	alanine transferase
ART	antiretroviral therapy
ATN	antiretroviral toxic neuropathy
AZT	azidothymidine (zidovudine)
B6	vitamin B6
BMI	body mass index
bp	base pairs
BPNS	Brief Peripheral Neuropathy Screen
cART	combination antiretroviral therapy
CCR5	C-C chemokine receptor 5
CXCR4	C-X-C chemokine receptor 4
d4T	2'-3'-didehydro-2'-3'-dideoxythymidine (stavudine)
ddC	2'-3'-dideoxycytidine (zalcitabine)
ddI	2'-3'-dideoxyinosine (didanosine)
d-drug	dideoxynucleotide reverse transcriptase inhibitor drug
df	degrees of freedom
DNA	deoxyribonucleic acid
dNTPs	dideoxynucleotides
DPM	DP Marais SANTA TB Hospital
DRG	dorsal root ganglion
DSP	distal sensory polyneuropathy
EDTA	ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtration rate
GOT	glutamic-oxaloacetic transaminase
gp120	glycoprotein 120
Hb	haemoglobin
HIV	human immunodeficiency virus
HIV-DSP	human immunodeficiency virus distal sensory polyneuropathy
HPLC	high pressure liquid chromatography
HR	hazard ratio
IENF	intraepidermal nerve fibre
INH	isonicotinic acid hydrazine (isoniazid)
INH-PN	isoniazid-induced peripheral neuropathy
IQR	interquartile ranged
MRC	Medical Research Council
mtDNA	mitochondrial deoxyribonucleic acid
MWM	molecular weight marker
n	number
NAT1	N-acetyltransferase 1 (enzyme)
NAT1	*N-acetyltransferase 1* (gene)
NAT2	N-acetyltransferase 2 (enzyme)
NAT2	*N-acetyltransferase 2* (gene)
NHLS	National Health Laboratory Services
NRS	numerical rating scale
NRTI	nucleotide reverse transcriptase inhibitor
OR	odds ratio
p	probability value
PCR	polymerase chain reaction
PGIC	patient global impression of change
PI	protease inhibitor
PI	protease inhibitor
PLP	pyridoxal 5‘-phosphate
p-m	person-months

PR	prevalence ratio
PTB	pulmonary tuberculosis
p-y	person-years
Q1	first quartile
Q3	third quartile
RANTES	regulated upon activation, normal T-cell expressed and secreted
RCT	randomised control trial
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RR	risk ratio
SD	standard deviation
SFN	small-fibre neuropathy
SNP	single-nucleotide polymorphism
SPNS	Subjective Peripheral Neuropathy Screen
TB	tuberculosis
TDF	tenofovir disoproxil fumarate (tenofovir)
TNF-α	tumour necrosis factor-α
TNS	Total Neuropathy Score
WCC	white cell count
WHO	World Health Organisation

Notes on terminology:

There is a lack of uniformity in the terminology referring to the various HIV/TB-associated sensory polyneuropathies. I have endeavoured to be consistent, and to utilise concise but descriptive terms. “Distal sensory polyneuropathy” (“DSP”) will refer to the generic clinical entity it describes (independent of aetiology) – DSP is more descriptive and specific than “peripheral neuropathy” or “sensory neuropathy”. DSP will always refer to symptomatic DSP, as opposed to “asymptomatic DSP” (“ADSP”) which implies the presence of neuropathic signs only. The terms, “human immunodeficiency virus distal sensory polyneuropathy” (“HIV-DSP”) and “antiretroviral toxic neuropathy” (“ATN”), are cited by our research group and I have maintained this nomenclature. In contrast, no specific term refers to the isoniazid-induced polyneuropathy – the description is generally one of a “peripheral neuropathy” or “peripheral neuritis”. I refer to this entity as “isoniazid-induced peripheral neuropathy” (“INH-PN”). The term, “HIV associated DSP”, will refer to primarily HIV-DSP and ATN, while “HIV/TB-associated DSP” will refer to primarily HIV-DSP, ATN and INH-PN.

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1 Introduction

The introduction presents an exposition of and approach to the research question.

1.1 Background

Distal sensory polyneuropathy (DSP) is a frequent and problematic complication associated with human deficiency virus (HIV) infection and tuberculosis (TB). DSP can be a consequence of the infection itself in the case of HIV-DSP, or as a result of treatment-related neurotoxicity: antiretroviral toxic neuropathy (ATN) and isoniazid-induced peripheral neuropathy (INH-PN). Nutritional, genetic and other factors may also be involved[1]. The risk for the development of DSP is increased by concurrent HIV and TB infection ("HIV/TB"), and during concomitant antiretroviral therapy (ART) and anti-tuberculous (anti-TB) therapy[2-5]. HIV/TB is frequently encountered and co-treatment necessitated in resource-limited settings, particularly in sub-Saharan Africa, because of overlapping HIV and TB epidemics[6,7]. In 2008, global incident TB was estimated at 9.4 million cases, and HIV co-infection was prevalent in 15% of these. However, South Africa bears the brunt of this problem: a disproportionate 28% of worldwide HIV/TB occurs in this country, and of the 0.38–0.57 million incident cases of TB in 2008, HIV co-infection was present in 70-73%[8,9].

HIV-associated DSP (implying HIV-DSP and ATN primarily), which is recognised as the commonest neurological complication seen in HIV infection[10], occurs in 49-57% of HIV-infected South Africans[11,12]. It presents a number of challenges to the clinician and patient. Clinically significant pain and discomfort, which occur at high frequency[11,13], may have a detrimental impact on quality of life[13-15]. Diagnostic difficulties arise from a wide differential and the inability to clinically distinguish aetiological processes – a delay in diagnosis, which is frequently missed, may lead to a subsequent intractable course[16]. Therapeutic choices are limited and poorly effective for neuropathic pain[10,17], which is under-treated[11]. A lack of preventative strategies[12] may result in the necessity to interrupt and substitute ART to alleviate DSP[18,19].

Neuropathic side effects have the potential to impact on treatment adherence[20,21].

A high background rate of HIV-DSP[11] is compounded by the use of combination antiretroviral therapy (cART) regimens containing neurotoxic nucleoside-analogue reverse transcriptase inhibitors (NRTI)s or “d-drugs”, such as stavudine (d4T). Despite World Health Organisation (WHO) recommendations to reduce and phase out the use of these agents, wide availability, low cost and limited alternatives in resource-limited settings ensures their continued use[12,22].

The risk of ATN due to d-drugs is associated with concurrent or previous anti-TB therapy[11,19]. Conversely, during anti-TB therapy, HIV-infection increases the odds for the development of INH-PN[4], the clinical features of which are markedly similar to those of HIV-associated DSP[16,23]. The causative agent, isoniazid (INH), is an integral component of first-line anti-TB therapy in South Africa, and elsewhere[24,25], and its antagonism of vitamin B6 (“B6”) metabolism induces a deficiency of the co-enzyme, which manifests clinically as a dose-related DSP in up to 44% of TB mono-infected patients on INH[23]. Adequate concomitant B6 supplementation in this population provides almost complete protection from this complication[26], but this preventative effect appears to be modulated by the presence of HIV, prompting the suggestion that co-infected patients may require higher doses of B6 supplementation[5]. An increased B6 requirement in HIV/TB has not yet been confirmed; however, current evidence demonstrates B6 deficiency present even in asymptomatic HIV infection[27], and in TB infection prior to initiation of anti-TB therapy[28]. Despite evidence demonstrating B6 deficiency in HIV/TB populations, B6 prophylactic coverage is poor in South Africa[11].

Another established modulator of the incidence of INH-PN is the rate of acetylation of INH, determined by polymorphisms in the gene coding for the hepatic enzyme, arylamine N-acetyltransferase 2 (NAT2)[29]. Slow acetylators, who may account for 40-60% of the population[30], are at a higher risk for the development of INH-PN[29], and have also been shown to exhibit impaired B6

function during anti-TB therapy[26]. While INH-PN is an established complication of anti-TB therapy, anecdotal reports suggest that TB itself can precipitate a DSP, both in TB mono-infected[31] and in HIV/TB co-infected populations – the latter possibly representing an unmasking of subclinical HIV-DSP[5,32].

In contrast to the knowledge base for INH-PN, which was studied extensively during the 1950s and 60s, our understanding of the pathogenesis of HIV-associated DSP is incomplete[10], with the literature presenting a complex interplay of various pathogenetic mechanisms[33]. The role of TB in these models is not entirely clear. Epidemiological data in HIV/TB populations is also lacking – current evidence is derived largely from retrospective studies in which DSP was not the primary outcome[2,3,5]. To date, there have been no published prospective studies specifically investigating the incidence of and risk factors for DSP in HIV/TB cohorts. Another deficiency in the literature is the lack of a consistent case definition for DSP. This bias may be addressed through the use of standardised instruments such as the Total Neuropathy Score (TNS) and Brief Peripheral Neuropathy Screen (BPNS)[34-36].

The prevalence of HIV-associated DSP remains high despite discontinuation of d-drug use in well-resourced settings, and is predicted to increase as life expectancy in HIV increases and additional age-related risk factors are accumulated[37]. ART penetration is still relatively low in resource-limited settings; as access is up-scaled, HIV-associated DSP is set to increase in frequency[38]. While the use of ART reduces the risk for TB, the incidence of TB has been shown to remain elevated at least five years after ART-initiation[39]. Even with full immune reconstitution, the risk for TB infection remains the same as in the general population[6], which is significant in sub-Saharan Africa[8]. Furthermore, there has been a drive by the WHO to institute INH preventive therapy globally[40]. HIV/TB co-infection and co-treatment rates can reasonably be expected to remain elevated, along with an associated DSP risk.

Vitamin B6 remains a justifiable target of study. Non-pyridoxine supplemented INH use results in INH-PN, and HIV/TB co-infection increases the risk for this complication, as well as for B6 deficiency. The optimum dose of pyridoxine in HIV/TB is not established[5], and B6 represents an affordable and simple potential intervention, with a minimal adverse effect profile if taken in appropriate dosages[1]. To inform the paucity of data regarding B6 needs in HIV/TB, a natural approach would be to assess B6 status in this population. Vitamin B6 status can be estimated by a number of direct and indirect assays; of these, ascertainment of plasma pyridoxal 5'-phosphate (PLP) concentration is currently considered the best indicator of whole body vitamin B6 status[41,42]. Enzymatic methods for direct estimation of plasma PLP have been used in recent studies of B6 status in relation to INH use[28,43], while earlier studies relied mainly on indirect methods[23,26,44]. Direct measurement of plasma PLP together with plasma 4-pyridoxic acid (4PA), a B6 degradation product, using high pressure liquid chromatographic (HPLC) techniques offers a more sensitive and precise method for vitamin B6 estimation[42], but these techniques have not been utilised in the study of B6 status in relation to INH.

Some authors have suggested that acetylation status inform INH dosing guidelines[29,45], but acetylation profiles have not been well characterised in the various South African populations. Acetylation status can be estimated by direct phenotyping and/or *NAT2* genotyping, but there is some uncertainty regarding the optimum method for ascertaining acetylation status[46]. Genotype is favoured, however, because phenotype can be unreliable in both HIV and TB infection[47-50].

High prevalence and incidence rates in a substantial population at risk; various diagnostic and clinical management issues; and a limited evidence base entreat the prioritisation of HIV/TB-associated DSP as a subject of research – targeted study of this clinical problem is needed. A prospective longitudinal approach would clarify the impact of various risk factors, offer a description of the natural history of HIV/TB-associated DSP and assist in delineating the relative contribution and interactions of contributing aetiological factors. Reinvestigation of the influence and interplay of the established

pathophysiological mechanisms for INH-PN (i.e. B6 deficiency and slow acetylation status), reframed within the context of HIV, and utilising modern validated methods, would help establish whether INH-PN is a primary contributor to HIV/TB-associated DSP, offer further insight into the driving forces behind this complication and possibly guide further research. The use of standardised and validated DSP instruments would reduce bias and allow for comparison and replication of study findings.

1.2 Research question

1.2.1 Primary (alternative) hypothesis

In HIV/TB co-infection, DSP is associated with B6 deficiency and/or slow acetylation status.

1.2.2 Secondary (alternative) hypotheses

In HIV/TB co-infection:

1. Markers of B6 status are lower in patients with DSP relative to those who are DSP-free;
2. Markers of B6 status are lower in slow acetylators;
3. DSP is more severe in B6 deficiency;
4. DSP severity is inversely correlated with markers of B6 status;
5. DSP is more severe in slow acetylators;
6. A relationship exists between DSP and dose and/or duration of INH therapy; the dose and/or duration of pyridoxine supplementation; and the use of cART (exploratory hypothesis); and
7. Markers of B6 status are correlated with the dose and/or duration of pyridoxine supplementation; and are inversely correlated with the dose and/or duration of INH therapy (exploratory hypothesis).

1.2.3 Aims

The study aims:

1. To characterise DSP in HIV/TB co-infection;
2. To establish risk factors for and pathogenetic features of DSP in HIV/TB co-infection with a focus on the role of vitamin B6 and acetylation status; and
3. To establish whether or not varying doses of B6 supplementation in HIV/TB co-infection are adequate for the prevention of DSP, thereby potentially informing the design of a future randomised control trial (RCT), and/or treatment guidelines.

1.3 Research approach

A suitable HIV/TB population in which prospective longitudinal study could be implemented was identified: co-infected inpatients at a TB hospital. Validated instruments and case definitions for the detection of DSP were selected: ≥1 neuropathic symptom plus ≥1 neuropathic sign by BPNS/TNS. Accurate, reliable and validated methods for the estimation of B6 and acetylation status were chosen: PLP/4PA by HPLC and *NAT2* genotyping, respectively. A research proposal and protocol were drawn up accordingly, and the specific objectives of the study were then:

1. To determine the baseline frequency and severity of DSP by BPNS/TNS in a population of HIV/TB co-infected inpatients at a TB hospital shortly after the initiation of B6-supplemented INH-containing anti-TB therapy;
2. To determine DSP incidence and the rate of worsening of baseline DSP during the period of admission;
3. To determine baseline and follow-up plasma PLP/4PA levels by HPLC, in order to determine the frequency and incidence of B6 deficiency and its association with baseline/incident/worsening DSP;
4. To describe *NAT2* genetic variation in the population and to predict acetylation phenotype accordingly;
5. To establish the association between *NAT2* genetic variation/predicted phenotype and baseline/incident/worsening DSP;

6. To determine whether a predicted slow acetylation phenotype is related to plasma PLP deficiency, and whether slow acetylators demonstrate lower PLP levels;
7. To determine whether higher doses of B6 supplementation are more protective for baseline/incident/worsening DSP over the standard dose;
8. To identify risk factors associated with baseline/incident/worsening DSP, and possible contributing aetiologies; and
9. To describe the natural history of baseline DSP.

2 Literature review

This review presents a summary of the current knowledge base for HIV/TB-associated DSP, with an emphasis on vitamin B6 and *NAT2* as pathogenetic risk factors. The review uses a standard clinical approach, namely: epidemiology, clinical features/diagnosis, pathogenesis and treatment/prevention. As the literature pertaining specifically to HIV/TB-associated DSP is limited, separate treatment of HIV-DSP, ATN and INH-PN was frequently necessitated.

2.1 Epidemiology

This section first contextualises then examines the epidemiological evidence for HIV/TB-associated DSP. Contributing HIV-DSP, ATN and INH-PN epidemiology data are also presented and briefly discussed.

2.1.1 Population

The population at risk for HIV/TB-associated DSP comprises individuals infected with concurrent HIV and active (as opposed to latent) TB: HIV+TB (**Figure 2-1A**). The respective therapies further define HIV/TB "sub-populations": (HIV+TB)+anti-TB, (HIV+TB)+ART and (HIV+TB)+anti-TB+ART (**Figure 2-1B**). In terms of the literature examining adverse events in HIV/TB, TB infection is often equated with anti-TB therapy, as the two are rarely studied independently, and anti-TB therapy often implies the use of INH, the primary aetiological factor associated with TB-related DSP[1]. ART presupposes HIV-infection, simplifying this approach further. Thus, the populations at risk for HIV/TB-associated DSP can be considered: HIV+INH (co-infection) and ART+INH (co-treatment) (**Figure** 2-1**C**). Frequently, HIV populations are mixed ART-treated and -untreated (i.e. not stratified), hence an additional category: HIV±ART+INH. Six papers inform the epidemiological evidence for HIV/TB-associated DSP within this population framework (**Table 2-1**). The population approach is laboured because it serves to contextualise the risk for DSP conferred by heterogeneous exposures in HIV/TB.

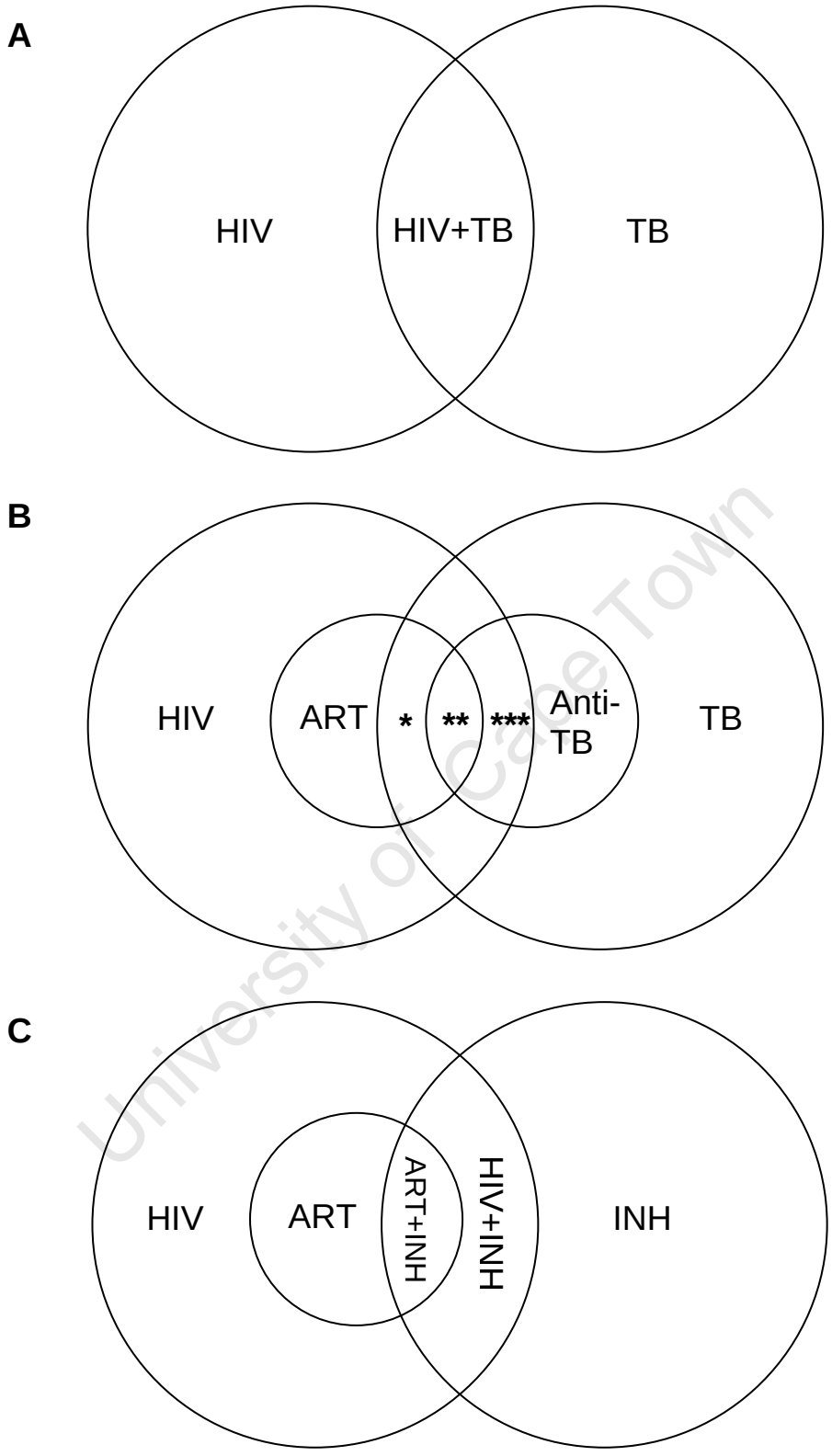


Figure 2-1 Venn diagrams illustrating HIV/TB populations at risk for DSP: (**A**) HIV+TB; (**B**) *(HIV+TB)+anti-TB, **(HIV+TB)+anti-TB+antiretroviral therapy (ART) and ***(HIV+TB)+ART (i.e. A + corresponding therapies); and (**C**) a simplified approach: HIV+isoniazid (INH) and ART+INH. Adapted from Wittes[51].

Table 2-1 Comparison of study design, study populations, DSP case definitions and pyridoxine supplementation among studies relating to HIV/TB-associated DSP.

	Study design	Populations*	DSP case definition	Pyridoxine
Perriëns et al.[52]	Randomised control trial	HIV+INH vs. INH	Presence of paraesthesiae	Not stated
Breen et al.[2]	Retrospective	ART+INH vs. ART	Not stated	Not stated
Dean et al.[18]	Retrospective	HIV±ART+INH	Not stated	Not stated
Breen et al.[3]	Retrospective	HIV±ART+INH vs. INH	Decrease in sensation to knees	10-25 mg/day
Lanternier et al.[4]	Prospective cohort	HIV±ART+INH vs. INH	"Clinically or electromyographically documented"	Unknown**
Marks et al.[5]	Retrospective	HIV±ART+INH vs. INH***	Decrease in sensation to knees	10 mg/day

ART=antiretroviral therapy, INH=isoniazid.
*See **Figure 2-1**.
**Pyridoxine was prescribed but supplementation status within the cohort could not be established because of technical reasons.
***Adults and children were studied.

Population categories do not remain static – for example, an individual can progress from HIV to HIV+INH to ART+INH: 25% of South African patients initiating ART are receiving anti-TB therapy[6]. Westreich et al. has modelled the chronological relationship of initiation of anti-TB therapy to ART: anti-TB therapy is "ongoing" if commenced prior to the initiation of ART, and "incident" anti-TB therapy is commenced after ("concurrent" anti-TB therapy is commenced within two weeks of commencing ART)[19] (**Figure 2-2A**). This model serves to orientate risks associated with the timing of treatment initiation, considered an important determinant of clinical outcomes[7,19]. The model is expanded in **Figure 2-2B** to better illustrate the temporal heterogeneity of exposures within HIV/TB populations. In this model, the population category defines possible DSP aetiologies, an approach utilised in the literature[4,5,11].

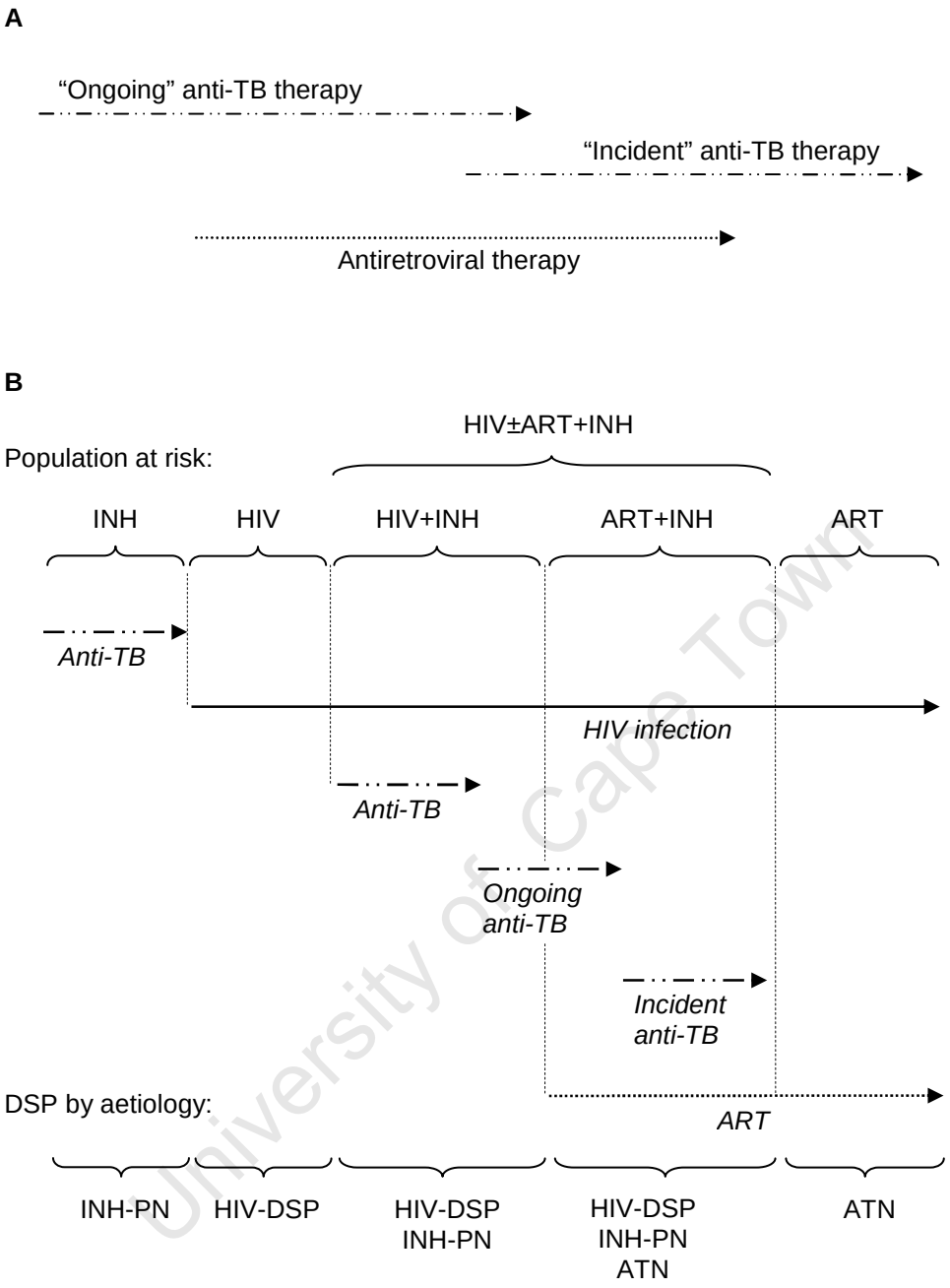


Figure 2-2 (**A**) Timeline relating relationship of anti-TB therapy to antiretroviral therapy (ART) as presented in Westreich et al.[19]. (**B**) Timeline relating relationship of populations at risk for DSP (see **Figure 2-1**) and aetiological factors contributing to DSP. INH=isoniazid, ART=antiretroviral therapy, INH-PN=isoniazid-induced peripheral neuropathy, HIV-DSP=HIV-distal sensory polyneuropathy, ATN=antiretroviral toxic neuropathy.

2.1.2 Incidence and risk in HIV/TB co-infection and co-treatment

DSP is cited as the most common adverse event during INH-containing anti-TB therapy in three reports of HIV/TB co-infected and co-treated cohorts[3,4,18]. The incidence rate of DSP in this population has been estimated

at 64.9 per 100 person-years[4]. In Perriëns et al., a RCT of extended anti-TB therapy conducted prior to the introduction of ART, the cumulative incidence of DSP in the HIV/TB co-infected (HIV+INH) group was 21%, as compared to 9% for the TB mono-infected (INH) comparator group[52]. In contrast, the post-ART data of Marks et al. presents a cumulative incidence of 8.3% for the HIV±ART+INH group vs. 1.9% for the INH group[5], and Breen et al. presents a cumulative incidence of 14% vs. 2%[3]. Neither of these studies stratified the HIV±ART+INH groups in terms of ART use. In Breen et al., DSP developed in a greater proportion of patients receiving INH/d4T versus comparator patients who received d4T but not INH[2]. **Figure 2-3** graphically demonstrates compounding DSP risks in these HIV/TB populations.

Multiple factors may contribute to the differences in the observed incidence among studies: inconsistent or absent case definitions for DSP, and lack of validated instruments; the retrospective nature of many of the studies; ill-defined study populations (no ART stratification, and in Marks et al., an unknown proportion of children); and varying pyridoxine supplementation regimens (**Table 2-1**).

Other evidence also points to increased DSP risk conferred by current or previous anti-TB therapy. In a cohort of ART-treated outpatients, the risk for withdrawal of d4T due to the development of DSP was 1.5 times higher in participants with incident or ongoing anti-TB therapy[19], and in another prospective cohort, TB at baseline increased the hazard for DSP during d4T-containing ART[53]. A history of previous TB has also been shown to be associated with DSP, increasing the odds for this complication in one cross-sectional study[11]; however, the association with previous TB has not been a consistent finding[12,22,54,55], possibly because of universal pyridoxine supplementation during INH therapy in some cohorts[54].

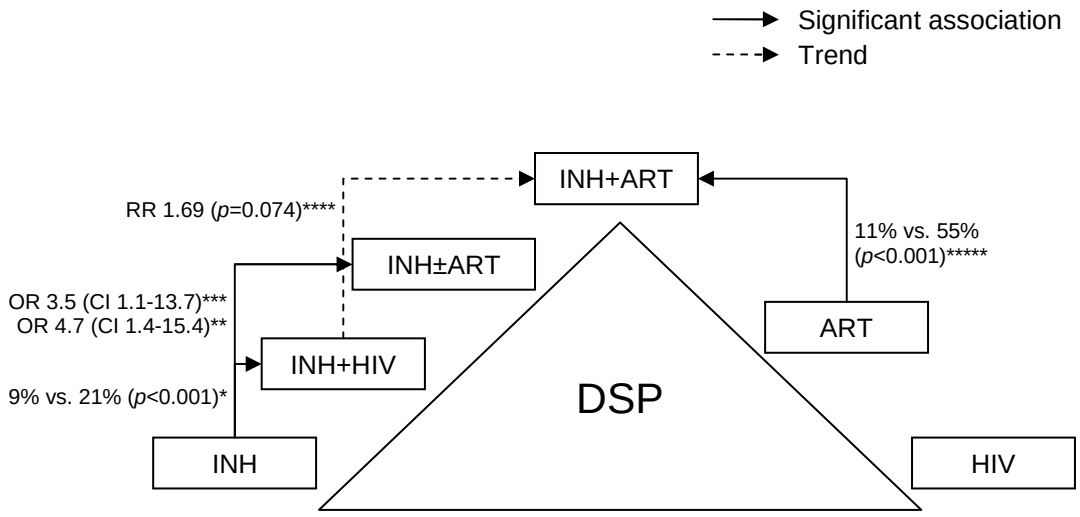


Figure 2-3 Schema of compounding risk for distal sensory polyneuropathy (DSP) in HIV/TV populations. Each step up from population to population represents increasing risk for DSP. On the left, additional risk for INH-PN is conferred by HIV and ART. On the right, risk for ATN is conferred by INH.. Based on and quoting the following evidence: *Perriëns et al.[52], **Lanternier et al.[4], ***Marks et al.[5] and Marks[56], ****Dean et al.[18], and *****Breen et al.[2]. ART=antiretroviral therapy, INH=isoniazid, OR=odds ratio, RR=relative risk.

2.1.3 HIV-DSP, ATN and INH-PN

Epidemiological data and associated risk factors for HIV-DSP, ATN and INH-PN individually are summarised in **Table 2-2**. These data should also be interpreted cautiously – multiple factors limit comparison and generalisability: selected study populations, heterogeneity regarding exposure to d-drug-containing regimens, use of varying diagnostic criteria (see Section 2.2.4.1), and variable periods of observation, among others[16,57-59]. These factors may also account for the often contradictory risk associations among studies. Mostly consistent risk factors for HIV-DSP are low $CD4^+$ T cell count and other markers of advanced HIV infection; for ATN, age and low *nadir* $CD4^+$ T cell count[16,59].

The incidence of treatment-related neurotoxicity is dose-dependent. Higher dose d-drug increases the risk for ATN when compared with lower doses[71,73,75]: for example, in Lichtenstein et al., d4T at 80 mg/day vs. 60 mg/day increased the odds for ATN[73]. In Biehl and Vilter,

Table 2-2 Epidemiological evidence and risk factors for INH-PN, HIV-DSP, ATN and mixed HIV-associated DSP.

	INH-PN	HIV-DSP	ATN	HIV-ass. DSP
Population at risk	INH	HIV	HIV+ART	HIV±ART*
Incidence	17/100 p-y[4]	No data	25/100 p-y[60] 11.9/100 p-y[61] 3.73/100 p-m[53]	No data
Cumulative incidence	2-44% (2-12 months)[23]	33-64% (±13 months)[62]	50% (±12 months)[63]	52% (12 months)[57]
Prevalence	No data	35%[64] 37%[11]	32%[54]	37%[11] 42%[58] 34.8%[13]
Risk factors**				
Age	---	++	+++/---	++
Gender	---			--
Male				
Female			++	
Weight		++/--		--
Height		--	+++/--	++/--
BMI		--	++/---	--
Race			+++/--	
Recent $CD4^+$	N/A	+++/--	++/---	---
Nadir $CD4^+$	N/A		+++/---	++/--
WHO stage IV	N/A		--	+++
Viral load	N/A		+++/---	--
HIV dementia	N/A	++		
Previous INH	+	--	+++/--	++/--
Alcohol	+/-	--	--	--
Diabetes mellitus	-	--	+++/--	--
Nutritional status	+/-			
D-drug exposure	N/A	N/A	+++/---	+++/---
Hepatitis C	---		++/--	--
Duration of HIV	N/A	+++	--	++
Duration of ART	N/A	N/A		
Increasing				++
Decreasing			+++	
PI exposure			+++/--	++

INH-PN=isoniazid-induced peripheral neuropathy, DSP=distal sensory polyneuropathy, p-y=person-years, p-m=person-months, $CD4^+$=$CD4^+$ T cell count, ART=antiretroviral therapy, BMI=body mass index, N/A=not applicable, PI=protease inhibitor
*Mixed cohorts comprising HIV-infected ART-naïve and ART-treated individuals (HIV±ART).
**Risk factor indicators: (+): associated; (-): no association; (+/-): conflicting evidence; ( ): no evidence. Best level of evidence: (+) and (-): anecdotal or no analysis; (++) and (--): cross-sectional or retrospective; (+++) and (---): prospective cohort or trial.
Evidence: INH-PN[65-69], HIV-DSP[11,62,64,70,71], ATN[12,53,54,61,72,73], mixed HIV-associated DSP[11,57,58,74].

2% of patients receiving INH at 3-5 mg/kg/day (without supplementary pyridoxine) developed INH-PN; at the much higher dosage of 16-24 mg/kg/day, the cumulative incidence was 44%. In this study, increasing dose was also associated with earlier presentation of INH-PN[23]. Low rates of

INH-PN in low-dose INH therapy is not a consistent finding, however. In patients presumed to be malnourished, 4-6 mg/kg/day INH produced INH-PN in 20%, suggesting the possibility that a poor baseline B6 status might predispose to overt deficiency[76]. Lack of a consistent case definition and the deliberate non-systematic elicitation of neuropathic symptoms may also account for the discrepancies – in the former study, symptoms may have been milder in the low-dose group, resulting in a response bias.

Duration of exposure to aetiological agents and duration of observation are other relevant considerations. For HIV-DSP, this is demonstrated in Husstedt et al., a prospective cohort of 42 ART-naïve patients: in just over a year, the frequency of DSP increased from 33% to 64%[62]. HIV-DSP incidence increases in tandem with HIV disease progression (as evidence by $CD4^+$ T cell count)[71], and nerve damage is evident in virtually all autopsy specimens of those dying from acquired immune-deficiency syndrome[16]. Originally, d-drug-induced neurotoxicity was thought to accumulate with ongoing exposure; however, after an initial peak at around three months, the incidence of ATN appears to decline, particularly after the first year of therapy[72,73]. Moreover, the use of d-drug-containing cART has in fact been found to be protective for DSP after the first year of therapy[13,22,73], suggesting recovery of neurological function in tandem with immune recovery[57,79]. However, this seemingly neuroprotective effect has been ascribed to survival bias (those at risk for ATN do so early in the course of therapy and are then switched to less neurotoxic agents, while those who are less at risk remain on neurotoxic therapy)[77,78].

The incidence of ATN during post-exposure prophylaxis is reported as 6% in one month[58]; the incidence of DSP in a group of mixed HIV-infected and -uninfected miners during pyridoxine-supplemented INH preventive therapy was 0.2% in 9 months[40].

2.2 Diagnosis and assessment

HIV-DSP, ATN and INH-PN share many clinical features which may preclude aetiological diagnosis in HIV/TB populations. Diagnosis is further hindered in both clinical and research settings by the lack of a formal case definition for DSP.

2.2.1 Clinical diagnosis

Characteristic subjective sensory symptoms and/or objective neuropathic signs in a distal symmetric lower-limb distribution inform the diagnosis of DSP. In advanced cases, there may be upper limb involvement[76,80]. Neuropathic signs in the absence of symptoms is termed asymptomatic DSP (ADSP).

2.2.1.1 Presentation

Distal symmetric and primarily lower limb pain, paraesthesiae and allodynia (positive symptoms), as well as numbness (a negative symptom), are the hallmark subjective sensory features of HIV-DSP, ATN and INH-PN[34,64,65,68,70,80-82]. Neuropathic pain is a prominent symptom in HIV-associated DSP, a primarily small fibre neuropathy (SFN)[83,84]. It is characterised as burning, stabbing and shooting[80], but is also variously described in terms of other unpleasant sensations[84].

2.2.1.2 Physical Examination

Examination findings reveal features consistent with both a SFN (impaired pinprick and temperature sensation) and a large fibre component (impaired vibration sensation and reduced ankle reflexes) in a length-dependant glove-and-stocking distribution. These features are similar for INH-PN, HIV-DSP and ATN[34,64,65,68,70,80-82]. Motor weakness, especially of ankle dorsiflexion, has been described for HIV-DSP[71,85], ATN[82] and INH-PN[69] in advanced cases but is never the predominant feature.

2.2.2 Differential diagnosis

In the general population, sensory polyneuropathy has multiple causes: for example, diabetes mellitus and glucose dysmetabolism; vitamin deficiencies;

chronic renal failure and other metabolic syndromes; neoplastic syndrome; and neurotoxic agents such as ethanol, among others. HIV/TB populations are likewise at risk for these polyneuropathies and their active exclusion in clinical settings is recommended[16,64,71].

HIV is also associated with a spectrum of non-DSP neuropathic disorders: acute and chronic inflammatory demyelinating polyneuropathies; mononeuritis multiplex due to cytomegalovirus infection (which also causes a progressive polyradiculopathy of the cauda equina), herpes zoster reactivation and myelopathies[10,84]. Mono-neuropathies due to nerve compression in poorly mobile patients can also occur[86]. A neurasthenic syndrome has been described in patients undergoing anti-TB therapy with INH[76], although this refers more to symptomatic fatigue.

2.2.3 Diagnostic investigations

Special investigations are useful to rule out alternative neuropathic processes, to elucidate specific nerve involvement and to quantify DSP progression, but do not serve to differentiate neuropathies of diverse aetiologies[16]. They are employed in some screening instruments and diagnostic criteria[34,87] (see Section 2.2.4); however, their use is not considered necessary in routine clinical practice[16].

2.2.3.1 Electrophysiology

Nerve conduction studies in HIV-associated DSP are often normal in predominantly SFN, but may reveal a sensory axonal neuropathy when there is large fibre involvement[84,85]. Decreased sural sensory nerve action potentials with normal distal latencies and velocities are typical findings[10,82]. Abnormal nerve conduction findings were associated with lower $CD4^+$ T cell counts in an untreated HIV-infected cohort, as were serum albumin levels and haemoglobin[88], probably reflecting the severity of HIV infection. Animal models of INH-PN demonstrate slowing of sensory conduction velocity within days of INH administration, followed by an increase in the duration of the compound motor action potential within weeks, without slowing of the efferent conduction velocity[89].

2.2.3.2 Pathology

Quantitation of intraepidermal nerve fibre (IENF) (C-fibre non-myelinated nociceptor) density in lower limb punch skin biopsies is considered an objective measure of HIV-associated SFN[84]. IENF quantitation involves specialised histochemical techniques; nevertheless, IENF quantitation has been employed as a reference standard for validation of DSP instruments[35] and as an outcome measure in drug trials[83], as well as clinically[84]. The primary findings are reduced IENF densities and features of degeneration[90]; IENF densities correlated with $CD4^+$ T cell count and inversely with plasma viral load in one study[35,83]. No reports of IENF quantitation in INH-PN appear in the literature.

Nerve biopsies are rarely performed in the diagnostic work-up of DSP because they are invasive[16], non-specificity limits their usefulness in differentiating aetiological processes[91] and pathological findings are not well characterised in ATN[10]. Wallerian/"dying back" axonal degeneration is a pathological finding common to all HIV/TB-associated DSPs, with loss of small and large myelinated fibres, as well as loss of unmyelinated fibres[85,86,92] – the latter is prominent in HIV-associated DSP[86]. Myelin abnormalities such as splitting and oedema were seen in rat models of ATN and INH-PN; myelin-containing macrophages were observed infiltrating peripheral nerves in the INH-PN model[93]. The presence of inflammatory cells, particularly activated macrophages, is a consistent finding in HIV-DSP important to its pathogenesis[10] (see Section 2.3.1.2). DRG neuron mitochondrial abnormalities are observed in ATN[86] (see Section 2.3.2.2.1) and also in rat INH-PN[94]. The pathology of HIV-associated DSP and INH-PN overlap, suggesting common underlying pathogenetic processes.

2.2.3.3 Quantitative sensory testing

Quantitative sensory testing is a computer-based technique quantifying sensory thresholds to vibration and hot and cold stimuli[80]. Temperature thresholds are a measure of small fibre function and correlate with IENF densities[84] – quantitative sensory testing findings in HIV-associated DSP

confirm a SFN[79]. Quantitative sensory testing has received criticism for having poor reproducibility[87] and are less suited to resource-limited and clinical settings[77,84].

2.2.4 Research diagnosis and assessment

Issues surrounding the research diagnosis and assessment of DSP relate to the establishment of a case definition and how best to measure the elements defining it, in light of the lack of a reference standard. Various composite instruments, comprising both clinical and investigative elements, which provide a quantitative appraisal of DSP status, have been developed to address these issues. Instruments range from single question screening tools such as the Subjective Peripheral Neuropathy Screen (SPNS)[80] to more involved composite scoring systems such as the TNS[34]. Many have been developed or adapted for HIV-associated DSP[35,80,95]: the BPNS (see Section 2.2.4.2) and modified TNS (see Section 2.2.4.3) have attained widespread use.

2.2.4.1 Diagnostic criteria and reference standards

Diagnostic criteria focus on the establishment of typical symptoms, signs and investigative findings in a distal symmetrical distribution[71,87]. The consensus evidence-based definition of DSP presented by the American Academy of Neurology concluded that emphasis should be placed on simple multi-criterion definitions which include assessment of a combination of neuropathic symptoms and signs, and that nerve conduction study should be integral to the case definition. A definition for small fibre neuropathy was deferred[87]. Benatar criticised the evidence informing this consensus statement for its predisposition to spectrum bias (the use of healthy controls, as opposed to controls with a risk profile similar to that of cases) and incorporation bias (when the instrument incorporates elements of the reference standard) which both artificially inflate sensitivity and specificity. The latter issue is partially a result of the lack of an independent gold reference standard[36] ("the best available method for establishing the presence or absence of the condition of interest"[96]). Similarly, proposed diagnostic criteria for SFN in Devigili et al.[84] may have suffered from spectrum and incorporation bias.

Lack of a gold standard not only limits development of a consensus DSP case definition, but also development and validation of screening instruments. Both have come to rely on multiple varied reference standards, such as neurologist assessment or quantitation of IENF densities, among others[35,80,95] (**Figure 2-4**).

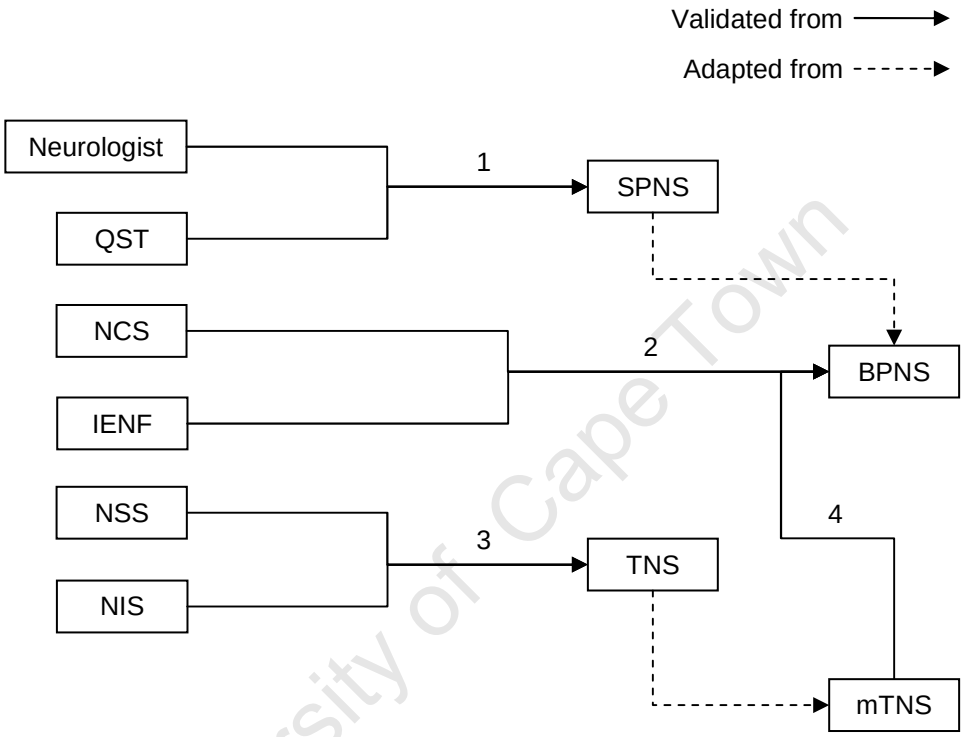


Figure 2-4 Schema of Brief Peripheral Neuropathy Screen (BPNS) and Total Neuropathy Score (TNS) validation studies. (1) McArthur[80]; (2) Cherry et al.[35]; (3) Cornblath et al.[34]; and (4) Ellis et al.[95]. SPNS=Subjective Peripheral Neuropathy Screen, QST=quantitative sensory testing, NCS=nerve conductions study, IENF=intraepidermal nerve fibre density quantitation, NSS=Neuropathy Symptom Score, NIS=Neurologic Impairment Score, mTNS=modified clinical TNS.

Instrument-specific diagnostic criteria include the widely applied ≥1 neuropathic symptom plus ≥1 neuropathic sign for the BPNS[12,55,74,97], which improves sensitivity[95] relative to the more stringent ≥1 neuropathic symptom plus ≥2 neuropathic signs[11,13,22,77]. The former definition was utilised in the validation of the BPNS[35]. ADSP may also be defined as ≥1 or ≥2 neuropathic signs, in the absence of symptoms. There are also TNS-specific criteria[77].

2.2.4.2 The Brief Peripheral Neuropathy Screen

The purely clinical BPNS is adapted from the SPNS (**Figure 2-4**), an early symptom screening tool validated for the assessment of HIV-associated DSP[80], Specifically, the symptom component of the BPNS is derived from the SPNS and is comprised of three 11-point numerical rating scales (NRS)s which patients use to rate symptoms of pain, paraesthesia and numbness from 0 (i.e. not present) to 10. In the BPNS, the highest NRS score (irrespective of specific modality) is used to establish a symptom grade. NRSs are widely used and well-established instruments for the measurement of pain[98]. The examination component of the BPNS includes assessment of ankle reflexes relative to knee reflexes and vibration sense at the big toe[35]. Though developed primarily for research purposes, the BPNS has also found clinical utility – a study of its acceptability to health practitioners found that its use impacted minimally on resources and time spent[61,97]. Two studies have validated the BPNS in HIV populations[35,95] (**Figure 2-4**).

2.2.4.3 The Total Neuropathy Score

The TNS was developed for the assessment of chemotherapy-induced neuropathy and validated in this population against the Neuropathy Symptom Score and Neurologic Impairment Score (**Figure 2-4**) with good intrarater and interrater reliability (0.984 and 0.938, respectively)[34]. The score is a composite of clinical assessments (neuropathic symptoms, reflexes, pinprick sensation, vibration sense and motor function), nerve conduction study and quantitative sensory testing. It has been criticised for the inclusion of time-consuming and expensive investigations[99] – exclusively clinical scales are considered more feasible in many research and clinical settings[100].

An attempt to remove these measures in order to simplify the TNS for HIV research purposes was presented in Evans et al. The analysis compared the full score with various combinations of TNS components (as well as EINF densities) using receiver operator characteristic curves. It showed that elimination of all TNS components other than quantitative sensory testing cooling detection, reflexes and pinprick sensation could be utilised in place of

the full score with acceptable sensitivity and specificity (85% and 80%, respectively). The authors also presented an algorithm for the diagnosis of DSP using the simplified score; however, because of the necessity of retaining at least one aspect of quantitative sensory testing, the objective of developing an exclusively clinical tool was not met[101]. Nevertheless, a purely clinical modified TNS has been utilised in several studies[11,63,95,102]. Importantly, the modified TNS retains pinprick sensation as a measure of small-fibre function. A modified 16-point[63] and 20-point TNS[11] score have been used as markers of HIV-associated DSP severity.

2.2.4.4 Symptom and single question screening

A single question screen as a means of further simplifying diagnosis is proposed in Robinson-Papp et al.[99] and Kandiah et al.[103]. In the former, "self-reported" pain and/or paraesthesiae predicted the presence of at least one abnormal objective item on the TNS with a sensitivity of 52% and a specificity of 92%. The low sensitivity was likely as a result of the high frequency of ADSP in the population. The high specificity supports the presence of pain and/or paraesthesiae as a useful rule-in test for the diagnosis of DSP; however, as the presence of symptoms was actively queried, unsolicited self-reported symptoms will not carry the same specificity – the use of the term "self-reported" in this study is therefore misleading. These findings contrast somewhat with those of Kandiah et al. in which a single question screen was compared to the BPNS with 96% sensitivity and 80% specificity[103]; however, incorporation bias might have played a role in inflating these data. The England et al. meta-analysis concludes that individual symptoms alone are poor indicators of DSP status[87], because of the frequent finding of a number of patients with isolated neuropathic symptoms (in the absence of neuropathic signs) (see Section 2.2.5.2).

2.2.5 Natural history

2.2.5.1 Onset

In isolated INH-PN, time to symptom onset is between 5 weeks and 6 months, and is dose-related – onset is earlier in patients receiving higher dose INH[23].

For HIV-DSP symptom onset is slow and insidious[60,82], with presentation 3-16 months relative to documentation of HIV infection reported in one study[85]. The median time to onset of ATN after initiation of ART is 6 months[75], with a peak at 3 months[60]. Compared to HIV-DSP, the onset of ATN is more abrupt[33,82].

In the HIV±ART+INH population in Lanternier et al., the mean time to onset of DSP after initiation of INH was 81 days (range 10-152 days)[4]. This is somewhat later than the median of two months reported in Dean et al.[18]. In Marks et al., DSP occurred entirely within the first four months of treatment, and this pattern of onset was similar in the INH only comparator group[5]. In the ART+INH population in Breen et al.[2], four months (range 2-15 months) is reported. (See **Table 2-1** for descriptions of these studies). The pattern of onset in these mixed populations is similar to that for the INH, HIV and ART only populations.

2.2.5.2 Progression

INH-PN is slowly progressive in terms of both symptoms and signs, both of which spread proximally[68]; paraesthesiae and numbness often precede painful symptoms[65,69]. This pattern has also been described in HIV-DSP[91]. In contrast, ATN progresses rapidly and pain is an early feature[91]. Later in the course of HIV-associated DSP, primarily positive symptoms evolve to primarily negative symptoms[83].

The review of Simpson and Tagliati suggests the possibility that ATN represents the unmasking of sub-clinical HIV-DSP[71], while Marks et al. suggests clinical unmasking occurs by TB-infection[5]. It has been postulated that ADSP precedes the full DSP-complex[33,79,97], but this has not been established: in a mixed cohort of ART-treated and -untreated individuals, ADSP did not predict incident DSP over 2.5 years of follow-up[57]. Pathological abnormalities have been noted prior to the onset of neuropathic symptoms[84,90], however. Purely symptomatic individuals do not differ from asymptomatic individuals in terms of non-clinical objective measures of DSP, such as IENF densities and quantitative sensory testing[35], and the

significance of isolated neuropathic symptoms as a clinical entity, or as a prodrome preceding demonstrable DSP, is yet to be determined.

Progression from SFN to a mixed fibre picture has been described in non-HIV/TB-associated DSP[84].

2.2.5.3 Reversibility

In Breen et al.[2], DSP is reported to have resolved after withdrawal of the d-drug component of the ART regimen; however, details regarding the rate and completeness of resolution are lacking for this observation, and further, the contribution of INH discontinuation at the end of anti-TB therapy to this reversibility is also unclear.

In HIV-only populations, ATN is also reported to resolve after withdrawal of the d-drug: ATN resolved after didanosine (ddI) was withdrawn shortly (3-5 weeks) after ART-initiation in one report[71]; however, ATN may worsen initially after withdrawal ("coasting"), and resolution of ATN may not be complete – neuropathic signs persisted over a year after zalcitabine (ddC) withdrawal in another study[33]. In a recent large meta-study of RCTs, symptomatic DSP persisted in over a third of participants after withdrawal of d-drugs. In this study increasing age was associated with decreased odds for recovery[74]. Similarly, INH-PN in TB-mono-infected populations resolves if INH is withdrawn soon after DSP onset, but prolonged use will result in a more protracted resolution, often with persisting neurological signs[23,67,104].

2.2.5.4 Distinguishing aetiological processes in HIV/TB

Onset or worsening of DSP following initiation of a d-drug, and subsequent improvement following d-drug withdrawal suggests ATN[91]. Natural history can therefore offer clues to an aetiological diagnosis in HIV-associated DSP. In prospective study, the onset of DSP is observed; however, patient recall of symptom onset has also been used to retrospectively make an aetiological diagnosis[54]. In cross-section, the distinction between HIV-DSP and ATN has been made simply by the presence or absence of ART exposure[11]. In HIV/TB-associated DSP also, the temporal association of DSP onset relative to

initiation of therapy has been employed: in Lanternier et al., DSP is attributed entirely to INH-PN[4], and in Breen et al., DSP is attributed to ATN[2].

2.2.5.5 Longitudinal DSP assessment

The study of DSP natural history contrasts with the longitudinal study of DSP incidence in that instruments are required that can detect changes in DSP severity over time; standard instruments such as the TNS and BPNS have not been validated for these purposes[34]. Nevertheless, the TNS has been used as a marker of DSP severity[54], and absolute differences in TNS scores have been utilised as measures of change in DSP status over time, both in HIV and non-HIV populations[77,100]. Serial nerve conduction studies[62] and quantitative sensory testing[79] have both been utilised to assess DSP progression.

Worsening of baseline DSP is an outcome measure in two longitudinal studies of HIV-associated DSP, albeit with contrasting but equally complex case definitions: Simpson et al.[77] and Hung et al.[22].

2.3 Pathogenesis

HIV/TB-associated DSP is a product of multiple intersecting pathogenic processes[1]. These are discussed under broad headings, with particular attention to the role of vitamin B6 and NAT2 acetylation. A proposed unified pathogenic model is also presented.

2.3.1 Direction infection and immune factors

Viral factors and immune responses are considered the primary pathogenic processes surrounding HIV-DSP.

2.3.1.1 Direct infection

HIV infection of nerve cell bodies in the dorsal root ganglion (DRG) has been demonstrated in autopsy specimens of patients with HIV-DSP[105]; however, this finding has not been consistent[86], and local infection is now thought to be limited to Schwann cells and infiltrating macrophages[33]. The TB bacillus is not known to infect neurons.

2.3.1.2 Viral toxicity and immune factors

The HIV envelope-derived glycoprotein 120 (gp120) induces cytochrome c and caspase-dependent peripheral nerve apoptosis and axonal degeneration[106]. The mechanism of neuronal injury in DRG cell bodies is indirect: gp120 binds to the C-X-C chemokine receptor type 4 (CXCR4) on Schwann cells inducing "regulated upon activation, normal T-cell expressed and secreted" (RANTES)-mediated neuronal tumour necrosis factor-α (TNF-α) release. TNF-α then binds to TNF receptors on the neuron, and, in an autocrine fashion, results in apoptosis[106] (**Figure 2-5**). Axonal injury may also be independent of cell body injury and Schwann cell chemokine release – gp120 will bind directly to axonal CXCR4 and C-C chemokine receptor type 5 (CCR5) causing axonal degeneration[107]. Direct axonal injury suggests that cell body apoptosis may be a process separate to that causing axonal degeneration[108], and may partially explain the distal length-dependent nature of HIV-associated DSP[107].

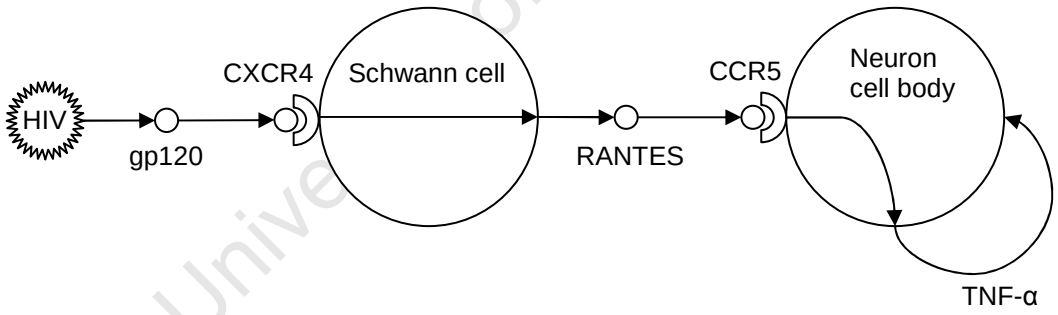


Figure 2-5 Model of glycoprotein 120 (gp120)-induced neuronal injury. HIV-derived gp120 binds to C-X-C receptor type 4 (CXCR4) on Schwann cells causing release of "regulated upon activation, normal T-cell expressed and secreted" (RANTES). RANTES binds to C-C chemokine receptor type 5 (CCR5) on neuronal cell bodies, inducing release of tumour necrosis factor-α (TNF-α) which acts in an autocrine fashion on neurons, causing apoptosis. Adapted from Keswani et al.[106].

Peripheral nerve gp120 may be derived from the circulation or secreted from infiltrating macrophages[106] – DRG infiltration by HIV-infected activated macrophages is a consistent finding in HIV-DSP[10,16] (see Section 2.2.3.2). Macrophages, whose activation is unopposed because of HIV-induced immune dysregulation[10,108], migrate to the DRG in response to gp120-

mediated neuronal injury[109] where they may further contribute to neuronal injury by further cytokine release[108], in addition to providing a source of gp120. Macrophage-derived cytokines also mediate neuropathic pain and hyperalgesia by binding to receptors on both injured and uninjured DRG cell bodies causing neuronal excitation or reducing the action potential threshold[109].

Concurrent gp120 and d-drugs may act synergistically to activate chemokine signalling pathways[110] suggesting that immune factors may play a role in the pathogenesis of ATN[109], in addition to those factors discussed in Section 2.3.2.2. Though macrophages have been demonstrated in models of INH-PN (see Section 2.2.3.2), their contribution to neuronal injury in this context is not known.

2.3.1.3 Inflammation

HIV and TB are inflammatory disorders[50,111], but the role of systemic inflammation in the pathogenesis of HIV/TB-associated DSP is not clear. Plasma TNF-α levels are elevated in TB[50] but their effect on neurons in this context is not known. Research in our group suggests that the incidence of ATN is not related to the elevation of any specific plasma inflammatory marker[112]. Systemic inflammation may represent an additional source of oxidative stress (see Section 2.3.2.2.2) and thereby contribute to the risk for DSP.

2.3.2 Pharmacotherapeutic agents

An array of pharmacotherapeutic agents may contribute to DSP in HIV/TB[1,91]; the focus here is on the role of INH and d-drug NRTIs.

2.3.2.1 Isoniazid

Isonicotinic acid hydrazide (hence “INH”), also known as isonicotinylhydrazide or isoniazid, is an anti-microbial agent with activity against mycobacteria currently used in combination anti-TB therapy. INH *monotherapy* has been used historically for the treatment of TB[26,65,68], and is presently employed as TB-preventive therapy in at-risk individuals[40].

The chemical structure of INH, which is characterised by a hydrazide functional group, is demonstrated in **Figure 2-6**.

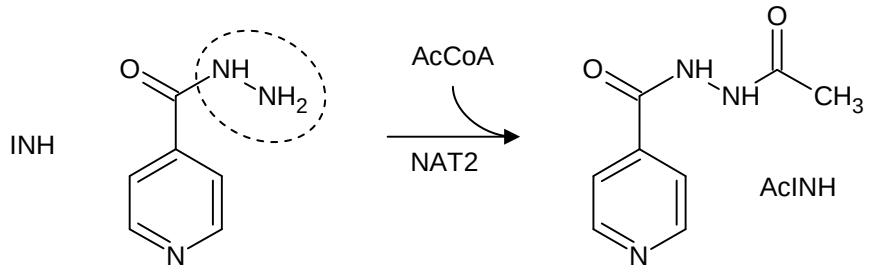


Figure 2-6 Acetylation of isoniazid (INH). The chemical structure of INH (with the hydrazide functional group highlighted) and the acetylated derivative, acetylisoniazid (AcINH), are shown. The conversion to AcINH is catalysed by N-acetyltransferase 2 (NAT2); acetyl coenzyme A (AcCoA) provides the acetyl group.

2.3.2.1.1 Metabolism of INH

INH is metabolised extensively in humans[113]: the primary pathway is acetylation of the hydrazide functional group by the xenobiotic metabolising enzyme, NAT2, yielding acetylisoniazid (AcINH) (**Figure 2-6**), but other pathways are also involved. The acetylation of acetylhydrazine (an AcINH derivative), to diacetylehydrazine, is also mediated by the NAT2 enzyme[114] (**Figure 2-7**).

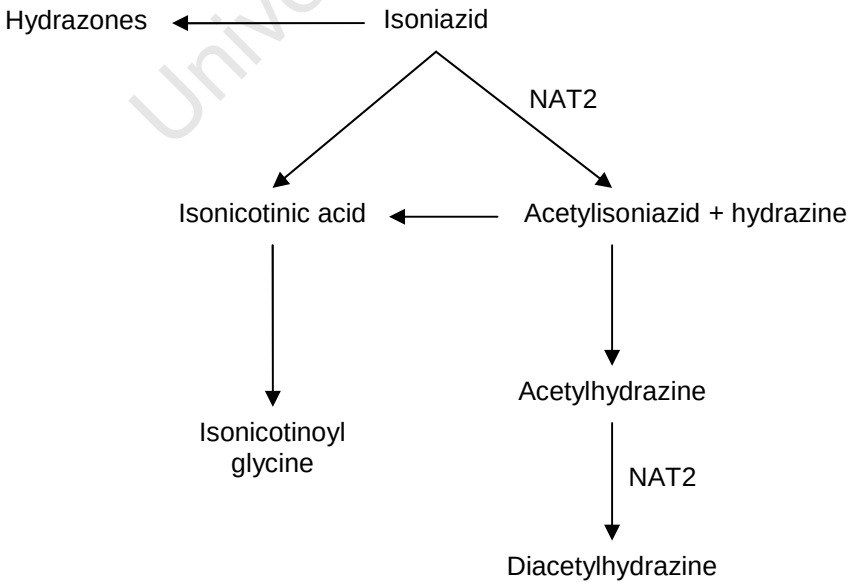


Figure 2-7 Isoniazid metabolic pathways. NAT2=N-acetyltransferase 2. Adapted from Preziosi[114] and Bryskier and Grosset[115].

NAT2, which is expressed primarily in the cytosol of liver and gastrointestinal cells, acetylates a wide array of xenobiotics, in addition to INH, including other hydrazine drugs and carcinogenic aromatic amines[114,116,117]. Acetylation in humans is also mediated by the structurally similar N-acetyltransferase 1 (NAT1) enzyme; however, NAT1 differs from NAT2 in terms of substrate affinity (although some overlap exists), and has a wider tissue expression[118]. NAT1 has not been implicated in the metabolism of INH[114].

The rate of NAT2 acetylation is variable among individuals within a particular population, as a result of variation in the highly polymorphic *NAT2* gene (see Section 2.3.3.1)[116,118]. The distribution of acetylation phenotypes within a population is trimodal – individuals can be categorised as phenotypically slow, intermediate or fast acetylators[119,120]; the proportion of individuals expressing each of these phenotypes differs from population to population. In Asian populations, the slow phenotype is rare (5-30%), while in European and African populations the slow phenotype is more frequent (40-90%)[30]. Acetylation phenotypes have been characterised in some South African populations: the mixed ancestry ("Cape coloured") population, which draws ancestry from Khoi-San, Asian, European and African population groups, demonstrates 35% slow acetylators[119] while a mixed population in Cape Town demonstrated slow acetylation in 18%[121]. Phenotype distributions in South African populations have also been estimated from genetic studies (see Section 2.3.3.1).

The acetylation phenotype results in differential risk for adverse drug reactions[118,119]. Slow acetylators receiving anti-TB therapy with INH reach higher INH plasma concentrations and are more prone to INH toxicity such as hepatitis and INH-PN that fast acetylators[26,45,68]. Fast acetylation is associated with lower INH concentrations, but acetylation phenotype has not been demonstrated to affect TB cure rates. However, the impact of this phenotype on therapeutic INH concentrations may be underestimated[119].

NAT2 acetylation phenotype can be determined by the administration of NAT2 probe drugs followed by the measurement of metabolite and unchanged probe concentrations in plasma or urine, the ratios of which (the "metabolic ratio") are used to estimate acetylation status[29,50,119]. The most commonly used NAT2 probe is caffeine, because of its relative safety and convenience, and because it is principally metabolised by NAT2[122]; this method has also been standardised and validated[123]. INH has also been utilised for acetylation phenotyping, because of its therapeutic relevance[119,120]. However, choice of metabolic probe may have an effect on the observed phenotype distribution and there is concern that phenotype classification is substrate-specific and not generalisable across the range of xenobiotics metabolised by NAT2[49,120]; for example, discrepancies between observed phenotypes have been demonstrated for caffeine and sulphadimidine within the same population[124].

A number of other factors may further influence observed phenotype. NAT2 activity is dependent on availability of the acetyl donor, acetyl-coenzyme A (acCoA), which may be depleted in certain clinical situations[46,125]. Reduced acetylation of a particular substrate may be a result of competitive inhibition of the enzyme by other xenobiotics; preferential acetylation of other substrates may also cause acCoA depletion[47]. However, exploratory data presented in O'Neil et al. did not support the hypothesis that acetylation of particular substrate is reduced by concomitant administration of other NAT2 substrates; further studies are required in this regard. Intra-individual variation in day-to-day caffeine acetylation capacity is 11-14.5% and could account for misclassification of phenotype in individuals whose metabolic ratios straddle the antimode[122]. Impaired renal clearance[45] might also account for phenotype variation.

Disease states can influence acetylation ability, as has been postulated and demonstrated for diabetes, TB and HIV. The rate of acetylation is faster in type 1 diabetics, presumably as a result of hyperglycaemia increasing the availability of acCoA[125]. In a recent study, 62% of TB mono-infected patients

on INH demonstrated a slow acetylation phenotype compared with 45% of healthy matched controls[50]. Slow acetylation phenotype was over-represented in patients with advanced HIV in one study, possibly because of genotype/phenotype discordance[48] (see Section 2.3.3.1.1), but in another study of less advanced infection, the distribution was normal[47]. Cascorbi et al. found no differences in acetylation capacity between hospitalised patients and healthy controls[122].

2.3.2.1.2 Effect of INH on vitamin B6 metabolism

Vitamin B6 refers to a group of several related compounds: pyridoxine, pyridoxal and pyridoxamine, along with their phosphorylated counterparts: pyridoxine 5'-phosphate, pyridoxal 5'-phosphate (PLP) and pyridoxamine 5'-phosphate. Their interrelation and common metabolic pathways are shown in **Figure 2-8**. The major metabolite of B6 metabolism is 4-pyridoxic acid (4PA), formed by the action of aldehyde dehydrogenase; it has no B6 activity and is excreted in the urine[126].

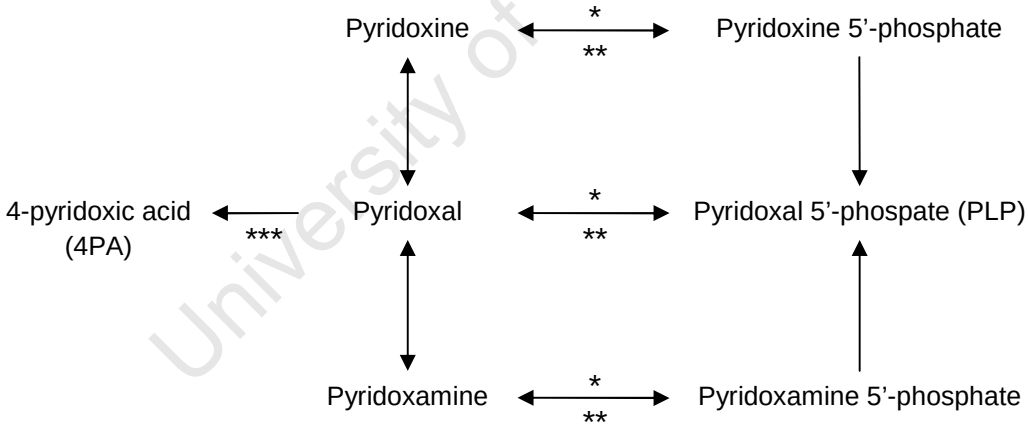


Figure 2-8 Pathways of vitamin B6 metabolism. The phosphorylation of pyridoxine, pyridoxal and pyridoxamine is catalysed by *pyridoxal kinase, while the reverse reaction is catalysed by **phosphorylases. All pathways, other than the formation of pyridoxic acid by ***aldehyde oxidase, are reversible. Adapted from Weiner and Klawans[127]; Brin[126]; and Meisenberg and Simmons[128].

The predominant active form, PLP, functions as a coenzyme in a wide range of metabolic reactions; transamination of amino acids and neurotransmitter synthesis are notable examples[127,128]. Synthesis of PLP is dependent on the phosphorylation of pyridoxal by pyridoxal kinase (**Figure 2-8**)[126] but its action is inhibited by INH, as well as by the complex formed between INH and

pyridoxal[26,114]. The inhibitory effect as evidenced by a reduced glutamic-oxaloacetic transaminase (GOT) activity, a functional assay of PLP status (see Section 2.3.4.1.3), can be demonstrated even in patients receiving pyridoxine-supplemented INH and who have elevated total B6 blood levels[26].

In addition to its effect on pyridoxal kinase, the pyridoxal-INH complex also interferes with the function of B6 as a coenzyme; however, the effect is probably apoenzyne-specific[127]. Complex formation may also reduce the amount of B6 available for use by tissues[114]. Urinary loss of the complex, which is excreted proportionally to the dose of INH administered, results in B6 deficiency[23]. The form of the pyridoxal-INH complex has been characterised as either pyridoxal isonicotinoyl hydrazone (a non-reversible imine) (**Figure 2-9**) or a Schiff base (a reversible imine)[114,129].

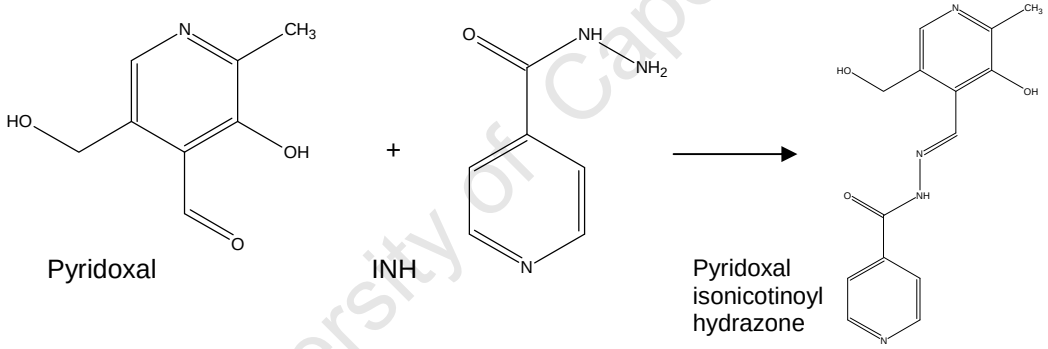


Figure 2-9 Irreversible formation of pyridoxal isonicotinoyl hydrazone from pyridoxal and isoniazid (INH).

Slow acetylators receiving INH demonstrate lower GOT activity than fast acetylators, but B6 concentrations do not differ between the two[26].

2.3.2.1.3 Neurotoxicity of INH

The neurotoxicity profile of INH is characterised by both central and peripheral phenomena. Central effects include seizures; restlessness and irritability; nervous system depression; and cognitive impairment, which is reversible. INH can also trigger psychosis[69]. Toxicity to peripheral nerves is manifested as INH-PN, the epidemiology and clinical features of which are discussed in Sections 2.1.3 and 2.2.1.

B6 deficiency is widely accepted to account for the peripheral neurotoxicity of INH[1,26,114] based on the observation that INH-PN occurs concurrently with a deficiency state[26], and that it can be prevented by and reversed on administration of pyridoxine supplementation[130] (see Section 2.4.1.2). Limited availability of B6 for use by neurons, with subsequent impaired neurotransmitter synthesis and impaired essential fatty acid metabolism, has been proposed as a possible mechanism of neuronal toxicity associated with B6 deficiency[94,114,127]. Another theory is that B6 deficiency disrupts ion gradients, thus accounting for the periaxonal swellings observed in histological specimens[94]. The predominant axonopathy of INH-PN supports the role of B6 – phosphorylation of pyridoxine occurs in the cell body and PLP is therefore more rapidly replaced proximally[94]. The precise mechanism of neurotoxicity associated with INH-induced B6 deficiency is still not conclusively established, however[1].

Slow acetylators experience INH-PN more frequently than fast acetylators (20% *vs*. 3% in Devadatta et al.) – presumably as a result of elevated plasma INH[26,68]. The role of NAT2 acetylation in reducing neurotoxicity is supported by the fact that the metabolite, AcINH, does not share the neurotoxicity profile of the non-acetylated drug[113].

The neurotoxic effects of INH may not be limited to those mediated by a functional and/or overt B6 deficiency. Hydrazines are toxic to dorsal root ganglia, plausibly as a result of free radical formation and resulting oxidative stress – *in vitro* neuronal cell death induced by hydrazine in one study was decreased in the presence of anti-oxidants, while pyridoxine was less protective[131]. Hydrazine toxicity may also account for the central nervous system effects of INH, in addition to other mechanisms, such as the inhibition of glutamic acid decarboxylase[127].

2.3.2.2 Nucleoside-analogue reverse transcriptase inhibitors

NRTIs form the backbone of most first-line highly active cART regimens[59]. Their antiviral capacity is exerted by competitive inhibition of viral reverse

transcriptase, and by the termination of viral ribonucleic acid (RNA) transcription after incorporation of the nucleotide analogue into the growing RNA strand[132]. NRTIs must be phosphorylated intracellularly before this can occur[133].

2.3.2.2.1 Effect of NRTIs on mitochondrial function

By similar mechanisms, NRTIs inhibit the function of mitochondrial polymerase-γ, the enzyme responsible for replication of mitochondrial DNA (mtDNA)[133]. The inhibition of polymerase-γ by NRTIs was initially thought to reduce mtDNA levels[134]; new evidence points to elevated levels, and the accumulation of mtDNA deletions and point mutations caused by increased turnover[135]. These mtDNA abnormalities cause defective structure and function of respiratory chain proteins leading to impaired oxidative phosphorylation, diversion to anaerobic metabolism and a rise in lactate[132,136]. Mitochondrial dysfunction also contributes to oxidative stress, the imbalance between the generation and clearance of reactive oxygen species, which can then cause further mitochondrial damage[136]. These mechanisms are thought to account for the various adverse effects associated with NRTI use: ATN, myopathy, pancreatitis, steatosis and potentially fatal lactic acidosis; a similar array of clinical manifestations are seen in inherited mitochondrial disorders[133].

2.3.2.2.2 Neurotoxicity of NRTIs

Of the NRTIs, the dideoxynucleosides (hence “d-drugs”), ddC, ddI and d4T, can cause ATN[33]. The relative neurotoxicity of the d-drugs, in order of decreasing toxicity is: ddC > ddI > d4T[137]. Consequently, the former agents, ddC and ddI, are no longer widely used in first-line cART regimens; however, d4T is still utilised in resource limited settings[22].

Various explanations are offered for the differential neurotoxic effects of the d-drugs. The energy-intensive metabolism of neurons relies mostly on aerobic energy production – processes that disrupt oxidative phophorylation will therefore have an effect on neuron function[132]. Another proposed mechanism is the differential phosphorylation of the nucleoside-analogues by various

tissue-specific thymidine kinase isoforms, and the affinity of the enzyme for any particular nucleoside analogue[133]. The absence of neurotoxic effects induced by other non-d-drug NRTIs is also accounted for: zidovudine (AZT), for example, does not cause ATN because its inhibition of mitochondrial polymerase-γ occurs at concentrations higher than those causing cellular toxicity[91].

That ATN is related to a mitochondrial cytopathy is supported by the observed association between elevated serum lactate and ATN[137] (although this has not been a consistent finding[11,77]), as well as the the presence of mitochondrial abnormalities in pathological specimens[134] (see Section 2.2.3.2). The axonal and length-dependent nature of ATN lends further support: mtDNA mutations increase distally as a function of distance from the cell body, the site of mitochondrial biogenesis[136], and height has been shown to be a risk factor for HIV-associated DSP, at least by some groups[12,55].

2.3.2.3 Other agents

Ethambutol, used in combination anti-TB therapy, can cause an optic neuritis in addition to sensory neuropathy in 4%. Cycloserine and ethionamide (both therapies for multidrug-resistant TB) are also associated with DSP, possibly through their interference with B6 metabolism[1]. Metronidazole, vincristine, steroids, dapsone, amphotericin B and phenytoin, agents that are used in the treatment of HIV-related illnesses, may all cause DSP[91]. Non-d-drug ART may be neurotoxic – a multivariate analysis in Ellis et al. found ART use predicted DSP even after adjustment for d-drug use[13]. Protease inhibitors may be implicated[73,74], but their association with DSP has been questioned[13].

2.3.3 Genetic factors

The *NAT2* gene and its relation to acetylation phenotype will be the main focus of this section. Several genes that influence the risk for HIV-associated DSP will also be discussed.

2.3.3.1 *NAT2*

NAT2 codes for the acetylating enzyme, NAT2, whose role in the metabolism of INH and the risk for INH-PN is covered in Section 2.3.2.1.1. *NAT2* is a

single-exon intronless gene that spans 870 base pairs (bp) and is located on chromosome 8p[116]. Variation in the *NAT2* gene is a result of single-nucleotide polymorphisms (SNP)s of which at least 25 have been reported[138]. The functional effects of frequently encountered *NAT2* SNPs have been determined using various *in vitro*, *in vivo* and *in silico* techniques[116] (**Table 2-3**). *NAT2* SNPs can occur in combination – to date, 66 haplotypes have been describedin Human *NAT2* alleles (haplotypes)[139] (available from: http://n-acetyltransferasenomenclature.louisville.edu/) utilising the standard nomenclature. *NAT2*4* is arbitrarily designated the wild type reference allele.

Table 2-3 Common *NAT2* single-nucleotide polymorphisms and associated amino acid changes, resultant functional effects and corresponding *NAT2** haplotype clusters.

SNP*	Amino acid Δ	Effect on NAT2 enzyme			rs ID	Cluster**
		Activity	Stability	Levels		
$G^{191}A$	Arg64 → Gln	↓	↓	↓	1801279	*NAT2*14*
$C^{282}T$	None	None	None	None	1041983	*NAT2*13*
$T^{341}C$	Ile114 → Thr	↓	None	↓	1801280	*NAT2*5*
$A^{434}C$	Glu145 → Pro	↓	None	↓	-	*NAT2*17*
$C^{481}T$	None	None	None	None	1799929	*NAT2*11*
$G^{590}A$	Arg197 → Gln	↓	↓	↓	1799930	*NAT2*6*
$A^{803}G$	Lys268 → Thr	None	None	None	1208	*NAT2*12*
$A^{845}C$	Lys282 → Thr	None***	None	None	5605745	*NAT2*18*
$G^{857}A$	Gly286 → Arg	↓	None	↓	1799931	*NAT2*7*

SNP=single nucleotide polymorphism, rs ID=restriction site identifier.
*Relative to the *NAT2*4* wild type allele.
**Each SNP defines a haplotype cluster in the standard nomenclature[139].
***Substrate-dependent.
Adapted from: Human NAT2 alleles (haplotypes)[139] (available from: http://n-acetyltransferasenomenclature.louisville.edu/), Hein[138] and Hein et al.[140].

NAT2 SNP and haplotype frequencies are population-dependent: for example, in African populations, the wild type allele is rare, whereas in European populations it is common[116]. Conversely, the $G^{191}A$ SNP is found in African populations, whereas it is not present in other populations[141]. Differences in haplotype frequencies account for the heterogeneity of acetylation phenotype distributions among populations[116] (see Section 2.3.2.1.1).

2.3.3.1.1 Prediction of acetylation phenotype

The prediction of phenotype based on genotype is dependent on establishing the presence or absence of SNPs known to code for NAT2 enzymes with reduced activity ("slow alleles") (**Table 2-3**)[138]. Phenotypic expression of *NAT2* alleles follows a co-dominant pattern – slow alleles are additive at each locus – resulting in three possible corresponding phenotypes: homozygosity for slow

alleles results in a slow phenotype, heterozygosity results in an intermediate phenotype and homozygosity for wild type alleles results in a fast phenotype[119]. However, a recessive pattern expressing a bimodal phenotype distribution, in which heterozygosity *or* homozygosity for a slow allele results in a slow phenotype, is cited widely in the literature[46]. Nonetheless, co-dominance corresponding to a trimodal phenotype distribution is based on clear evidence, albeit under specific experimental conditions, and is the currently accepted view of *NAT2* expression[119,120]. It should be noted, however, that one study failed to re-demonstrate a trimodal distribution under similar conditions[69], and phenotype distribution as related to genotype may be population-specific[46] or substrate-specific[124] (i.e. trimodal for one particular population or substrate, bimodal for another). A "gene-dose" effect in which the *total* number of mutated alleles (slow and non-slow) are additive has also been proposed, but probably requires further exploration[142].

The concordance rate between actual phenotype and that predicted by genotype is 94-100%[47,48,119]. However, a number of caveats relate to the determination and prediction of acetylation status by both phenotyping and genotyping methods which may contribute to an apparent discordance[138] (**Figure 2-10**). Discordance can result in failure to identify disease association, and non-replication of findings[138].

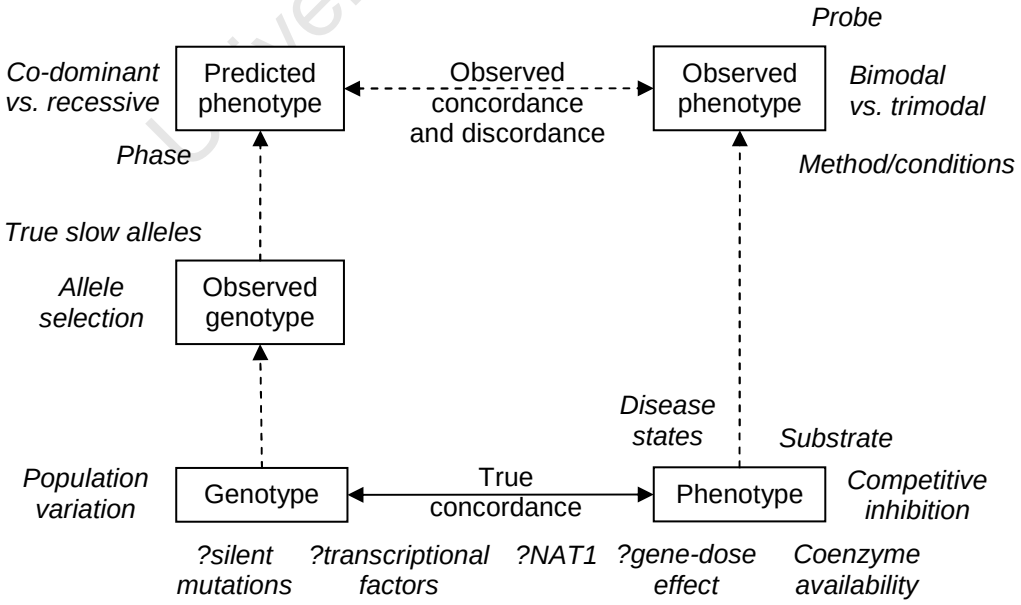


Figure 2-10 Schema of factors influencing concordance and discordance between predicted and observed acetylation phenotype.

Prediction of phenotype rests on the determination of slow alleles present in the genotype, but the assumption that a particular allele is slow or fast may not be true, resulting in discrepancies between observed and predicted phenotype[123]. Assuming that alleles defined as slow are in fact true slow alleles, failure to type any of these alleles, particularly if frequent in a given population (for example, the G[191]A in Hispanics, Arabs and South Indians), will result in bias towards non-slow predicted phenotypes[46]. Population differences in predicted phenotype distribution among studies have been attributed in part to differences in the completeness of genotyping[48].

Another caveat in the literature is the failure to recognise a co-dominant expression pattern and resulting trimodal distribution of acetylation phenotype in the population – some instances of genotype/phenotype discordance, for example in Bolt et al.[123], are readily explained by the trimodal model.

Polymorphisms in non-coding regions may a play a role in *NAT2* transcription, translation, expression and phenotype. Polymorphisms in the *NAT2* promoter region have been identified; however, their functional significance has not been characterised[143,144]. No *NAT2* epigenetic factors are known[46]. Silent *NAT2* mutations, such as the C[282]T, do not alter the amino acid sequence and are therefore not assumed to have any functional effect (**Table 2-3**); however, this concept has been challenged by findings that suggest that silent mutations may affect codon usage and messenger RNA folding, and thereby alter protein translation and function[145]. Although probably not playing a role in INH acetylation, NAT1 has been shown to overlap somewhat with NAT2 in terms of substrate-specificity, and is also subject to genetic variation – failure to account for the contribution of NAT1 to acetylation may be a factor leading to discordance[116].

When heterozygous slow alleles are present at two or more loci, the genotype is said to be ambiguous and phenotype prediction requires the determination of the phase of each allele – if located on the same chromosome, the opposite chromosome will carry no slow alleles and the predicted phenotype will be intermediate; if located on opposite chromosomes, both copies will be

mutated[116]. Phase can be resolved by parental genotyping and other molecular or computational techniques[146].

Apparent discordance between observed and predicted phenotype may be substrate-dependent: discordance has been shown to be greater for caffeine than for dapsone[49,138]. Such discrepancies may limit the utility of both genotyping and phenotyping tests in disease-association studies[124].

Finally, discordance was demonstrated in a cohort of moderately advanced HIV patients wherein 24% of those with a fast predicted phenotype were found to be slow acetylators on caffeine phenotyping, and 12% demonstrated the reverse (slow to fast)[49]. In this study, markers of immune suppression did not predict discordance; however, patients with less advanced HIV do not demonstrate discordance[47].

Other factors that influence the observed phenotype and thereby apparent concordance with genotype are discussed in Section 2.3.2.1.1.

2.3.3.1.2 NAT2 and DSP risk

NAT2 genotype (as opposed to phenotype) has not been studied extensively in relation to the incidence of INH-PN. In Hiratsuka et al., an INH-mono-treated cohort, all of two cases of INH-PN occurred in genotypically slow acetylators; however, as the INH-PN group formed part of the total adverse event group in the analysis, genotypic risk for INH-PN was not established. It is worth noting that, in this study, predicted phenotypes corresponded with metabolic ratios, but not with INH concentrations (see below). The observed association between acetylation and INH-related adverse reactions may be attributable to a metabolic process unrelated to absolute INH concentration[45].

NAT2 as an independent risk factor for HIV-associated DSP is currently being investigated in our group.

2.3.3.2 Other genetic factors

The variably penetrant apolipoprotein E4 allele, which has received attention in the literature mostly regarding its role in modulating the risk for central

nervous system disease, was shown to be associated with HIV-DSP in a small longitudinal study[147]. Delayed peripheral nerve regeneration, as demonstrated in mouse models[148], may account for the association.

The presence of mitochondrial haplogroup T in white patients receiving either ddI plus d4T-based or AZT plus lamivudine-based cART regimens was demonstrated to independently increase the odds for ATN; the odds were further increased in those receiving the former regimen (i.e. two d-drugs), reflecting an increased mitochondrial susceptibility to mitochondrial-toxic agents conferred by this haplogroup. The frequency of this allele in the white population is estimated at 10-15%[149].

In the same cohort, heterozygosity for the $G^{845}A$ mutation in the haemochromatosis *CFE* gene was shown to independently decrease the odds for ATN induced by d-drug use; the odds were further reduced by limiting the analysis to white participants. Ten percent of the white population are heterozygous carriers of the mutation[150].

The *TNFA-1031*2* allele, coding for a variant TNF-α protein, has also been shown to increase the risk for ATN[54]. A mutation of the mitochondrial polymerase-γ gene could increase susceptibility to d-drug-induced mitochondrial toxicity[151], although an association with ATN has not yet been demonstrated.

2.3.4 Metabolic and nutritional factors

The most clearly defined nutritional factor relating to HIV/TB-associated DSP is vitamin B6 deficiency; its association with INH-PN is reviewed in Section 2.3.2.1.3. The possible contribution of dietary B6 deficiency to DSP, as well as the role of other metabolic and nutritional factors, are discussed here.

2.3.4.1 Vitamin B6

2.3.4.1.1 Dietary source and deficiency

Vitamin B6 is obtained from the diet mainly as pyridoxine and its glucoside derivative. Major sources are cereals, animal products and non-citrus fruits;

offal is particularly B6-rich. Absorption of B6 takes place by passive diffusion primarily in the jejunum, and the bioavailability of dietary B6 is about 75%[126,152,153]. Markers of B6 status correlate with dietary intake, particularly as intake levels approach those approximating that obtained from pyridoxine supplementation[27].

B6 deficiency is rare in the general population, and when present is usually a component of a multivitamin deficiency[130]. At risk are pregnant women, the elderly, alcohol abusers and those with bowel and renal disease[130,153,154]. Both HIV-infection and TB are associated with B6 deficiency, irrespective of concurrent treatment status. In one study, ART-naïve males with WHO stage I HIV disease had significantly reduced markers of B6 status when compared with matched controls, and over a third demonstrated overt deficiency, despite adequate dietary B6 intake and without clinically demonstrable deficiency[27]. In a cohort of 20 South African TB-infected patients, five of whom were HIV/TB co-infected, B6 deficiency was universal prior to the start of anti-TB therapy[28]. In a cohort of children receiving INH (along with 0.5-1.0 mg/day supplementary pyridoxine), 50% of those who were HIV-infected were B6-deficient, compared with 15% of those who were HIV-uninfected[43]. Ongoing research in our group demonstrates that HIV-infected individuals are B6-deficient before and after commencing cART: of 159 patients in our outpatient cohort, almost all of whom received vitamin B-complex, 51% were B6-deficient at baseline, and 54% after three months of cART[112].

Proposed mechanisms of B6 deficiency in HIV-infected individuals with adequate dietary B6 intake include malabsorption from enteropathy, renal losses and impaired B6 metabolism from hepatic insufficiency[27]. Another possible mechanism is inflammation, which is associated with B6 deficiency in rheumatoid arthritis[155]. A similar association may be implicated in HIV-infection and TB, as systemic inflammation is prominent in both[50,111]. The acute phase response has been proposed to explain the presence of B6 deficiency in TB-infected individuals prior to anti-TB therapy[28]. This is based on evidence that deficiency demonstrated in healthy patients undergoing orthopaedic surgery is attributable to the acute phase[156]. The association may

be explained by a concurrent drop in albumin, a known acute phase indicator[154,156], and the primary B6-carrier protein (see Section 2.4.1.2.3). Hypoalbuminaemia is also common in both HIV-infection and TB[157]. Finally, as muscle is the primary store of B6 in the body[155], muscular wasting, also common in HIV/TB[157], could be implicated.

2.3.4.1.2 The role of dietary B6 deficiency in DSP

There are no descriptions of DSP resulting from a pure dietary B6 deficiency (although central nervous system effects are seen in severe deficiency states)[130,158] which may suggest that pure dietary deficiency is not an important contributory factor to HIV/TB-associated DSP. However, pre-existing B6 deficiency could well be exacerbated by INH administration, increasing the risk for DSP, and the need for supplementation. This has been proposed in Marks et al.[5] but has not been shown. Possible explanations for the discrepancy in risk for DSP between INH-induced and dietary B6 deficiency include a B6-independent INH neurotoxicity[114] (see Section 2.3.2.1.3), and the complex metabolism of B6 being affected differentially by each aetiological process, with differing resultant phenotypes[127]. Inflammation and immune dysregulation contribute to HIV-associated DSP (see Section 2.3.1) and are also associated with B6 deficiency[27,156] – the association might be causal. Diabetics with neuropathy demonstrated lower serum pyridoxal when compared to diabetics without neuropathy in one study[159], but a follow-up RCT failed to show a benefit of pyridoxine supplementation in alleviating diabetic neuropathy[160].

2.3.4.1.3 B6 status estimation

In humans, B6 status can be assessed by various assays performed on blood, plasma/serum and urine samples, and are generally directed at estimation of the circulating active coenzyme, PLP, currently considered the best approximation of whole body B6 status[42,155]. The assertion that PLP alone is an adequate estimate has been challenged, however[41] – the recommendation in Rybak and Pfeiffer is to evaluate both PLP and 4PA[42].

Table 2-4 Indirect and direct assays of vitamin B6 status.

Assay	Biochemical pathway	Method	Notes
Indirect methods			
Tryptophan load test	Inhibition of PLP-dependent kynureninase diverts tryptophan catabolic pathway to xanthurenate synthesis	Measurement of xanthurenate in urine by chomatographic methods following an oral load of 2 grams of tryptophan	Most widely used indirect test Relatively non-specific
Methionine load test	Inhibition of PLP-dependent cystathionine synthase in methionine catabolic pathway	Measurement of urinary cystathionine following an oral load of 3 g of methionine	Reference values not established
Erythrocyte transaminases (GOT, EAST, etc)	Inhibition of PLP-dependent aminotransferases	Measurement of the activity of erythrocyte alanine or aspartate transaminases before and after stimulation with excess PLP and expressed as a ratio	Indicator of long term status - delay in reflecting recent changes in B6 intake Lack of standardisation
Tyrosine decarboxylase test	Inhibition of PLP-dependent tyrosine decarboxylase	Detection of radio-labelled CO_2 released by the activity of tyrosine decarboxylase	Currently employed indirect assay
Direct methods			
Total B6		Microbiological assay	No longer in use
Pyridoxal 5'-phosphate	Primary active form	Chromatographic methods Measured in plasma	Considered best indicator Reference range >30 or >20 nmol/l
4-pyridoxic acid	Endpoint of B6 metabolism – represents turnover	Chromatographic methods Measured in urine or plasma	Represents recent exposure to pyridoxine
Pyridoxal	Primary transport form	Chromatographic methods	Not commonly employed

PLP=pyridoxal 5'-phosphate, GOT=glutamate oxeloacetate transaminase, EAST=erythrocyte aspartic acid transaminase.
Adapted from: Shane[162], Ubbink et al.[163], Leklem[41], Rybak and Pfeiffer[42] and Cilliers et al.[43].

Several indirect and direct methods of B6 estimation have been developed. Indirect methods are functional assays that estimate PLP concentration by the measurement of apoenzyme activity both *in vivo* and *in vitro*[41] (**Table 2-4**), and have been utilised in much of the B6-related research presented in this

review[23,26-28,43,155]. However, these methods are imperfect and conflicting[26,44,161].

Direct estimation of B6 is achieved through microbiological and chromatographic assays. The former measures total B6[26]; the latter allows independent quantitation of the different B6 fractions, including 4PA[42,153]. Refinement of HPLC methods has resulted in sensitive and precise estimations of B6 status[42].

2.3.4.2 Malnutrition and other vitamin deficiencies

Malnutrition is frequent in HIV/TB populations; several micronutrient deficiencies have been described of which several are known to cause syndromes with DSP as a component. Deficiencies associated with TB are thiamine, folate and vitamin E; these are also seen in HIV, along with B12 deficiency[153,157]. The latter and its relation to HIV-associated DSP was studied in a selected sample of HIV-infected patients on AZT: a third with DSP demonstrated B12 deficiency compared to none without DSP. In the same study, improvement in neurological signs occurred in 5/8 receiving B12 replacement, thereby strengthening the association and the assertion that B12 deficiency is contributory to HIV-associated DSP[164]; however, there is some doubt about the relevance of this evidence[153]. Reduced antioxidant activity in vitamin E deficiency may also play a contributory role[165].

While it is conceivable that various vitamin deficiencies can contribute to HIV/TB-associated DSP, the extent of the contribution is not known[59].

2.3.4.3 Other metabolic factors

The metabolic syndrome, defined as the presence of three or more of hypertension, dyslipidaemia, dysglycaemia and obesity, was an independent risk factor for small fibre neuropathy in a retrospective study of a mixed neuropathy population[166]. Similarly, elements of the metabolic syndrome (hypertriglyceridaemia and diabetes) are associated with HIV-associated DSP[11,37]. HIV infection and ART use can increase risk for the metabolic syndrome, possibly as a result of inflammation and lipid abnormalities, while

an increasingly ageing ART-treated population may also contribute by accumulation of metabolic risk factors[37]. Alcoholism is a known cause of neuropathy, most likely as a result of a multivitamin deficiency, and is therefore a plausible contributory factor to HIV/TB-associated DSP[153]; however, the evidence does not reflect this (**Table 2-2**). HIV-infected individuals suffering with DSP may turn to alcohol for relief of symptoms[167].

2.3.5 Model

It is evident that HIV/TB-associated DSP pathogenesis is multi-factorial. The prevailing hypothesis is that peripheral nerve injury is cumulative over time and each neurological insult is contributory to the final clinicopathological phenotype, and to the final clinically relevant endpoint of DSP: pain[19,33,71]. **Figure 2-11** presents a hypothetical model based on that in Cherry et al.[33], and is a synthesis of the evidence for HIV/TB-associated DSP presented in this review.

2.4 Treatment and prevention

Neuropathic pain responds poorly to analgesia[17], and in HIV, impairs quality of life[13-15] and is associated with maladaptive behaviours[21,168]. Both pharmacological and non-pharmacological approaches to the treatment and prevention of DSP in HIV/TB are explored in this section.

2.4.1 Pharmacological therapies

Treatment of HIV-associated DSP is largely symptomatic, as there are currently no disease-modifying agents[16], though cytokine receptors are a promising therapeutic target[109]. Oral pyridoxine supplementation is an established prevention and treatment strategy for INH-PN.

2.4.1.1 Symptomatic treatment of neuropathic pain

HIV-related pain is under-treated in a South African setting[169]; HIV-associated DSP is a notable example – in one study, under 7% of symptomatic individuals were receiving analgesia for their neuropathic pain[11]. Under-diagnosis, under-estimation and under-treatment are recognised issues in the clinical management of HIV-associated DSP[16].

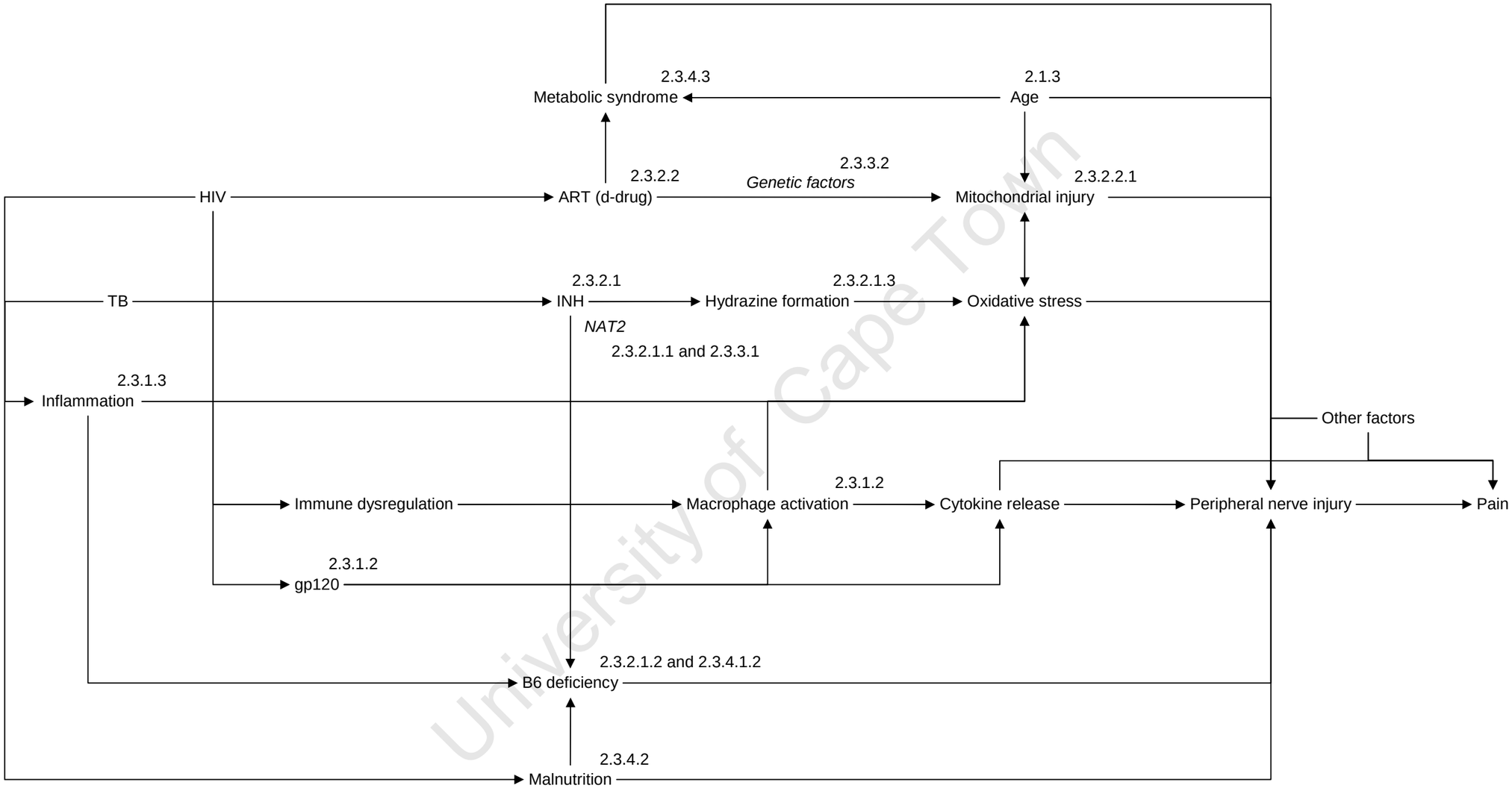


Figure 2-11 A hypothetical model of HIV/TB-associated DSP pathogenesis. Each interaction is referenced to the relevant section of this review. Based on Cherry et al.[33].

Three major classes of pharmacotherapeutic agents are used for symptomatic relief of neuropathic pain: antidepressants (amitriptyline, duloxetine, venlafaxine), anti-epileptic drugs (carbamazepine, gabapentin, lamotragine, pregabalin) and topical agents (capsaicin, lignocaine)[16]. Non-steroidal anti-inflammatories, paracetamol and opioids are also used to treat neuropathic pain, but are unproven[16,71].

The tricyclic antidepressant, amitriptyline, which is commonly prescribed for neuropathic pain in South Africa[19], is poorly effective[170] – a recent meta-analysis presented a numbers needed to treat (NNT) of >10 for HIV-associated DSP. Pregabalin had a similar NNT in this analysis while lamotrigine fared better (NNT=~5), as did topical capsaicin (NNT=6.5)[17]. Pregabalin is considered a promising agent but was found to be mostly ineffective in an RCT; methodological issues in this study might have contributed to the negative findings[78]. An international multi-centre trial reinvestigating this agent is currently under way.

2.4.1.2 Pyridoxine

2.4.1.2.1 Indications

In individuals with a low risk profile for B6 deficiency or DSP, it is not considered necessary to give supplementary pyridoxine routinely during standard dose INH therapy – the incidence of INH-PN in this group is extremely low[130]. In high-dose INH therapy, as employed in the 1950s, the addition of pyridoxine should prevent the majority of INH-PN cases that would be expected in unsupplemented therapy at similar INH doses[23,26]. Based on both observation[76,104] and clinical prudence[130], pyridoxine supplementation is recommended in high risk groups (see Section 2.3.4.1.1), even during low-dose INH therapy. However, HIV is not mentioned as a high risk group in the South African TB management guidelines[24] in contrast to international guidelines and the South African Medicines Formulary which recommend prophylaxis in this population[1].

Once INH-PN is established in unsupplemented anti-TB therapy with INH, treatment with pyridoxine can be effective[67]; earlier pyridoxine therapy is associated with a better neurological outcome[26,67].

It is not known whether pyridoxine supplementation has any impact on the incidence of HIV-associated DSP (in the absence of INH therapy), and whether it might have any therapeutic value – our group is currently investigating the role of vitamin B6 in HIV-associated DSP[112].

2.4.1.2.2 Dosage

Consensus is lacking in terms of the optimum dose of prophylactic pyridoxine; dosing guidelines are inconsistent with recommended doses ranging from 10-50 mg/day[1,171]. Pyridoxine needs are probably a function of INH dose[104] but doses as low as 6 mg/day have been demonstrated to prevent INH-PN during high-dose INH in TB mono-infected[26]. In HIV/TB co-infection, 10 mg/day pyridoxine failed to prevent incident DSP as reported in Marks et al.[5]; in light of pre-existing B6 deficiency (see Section 2.3.4.1.1) there is value in reinvestigating B6 needs in this group.

Recommended dosages for treatment of INH-PN are usually higher than those for prophylaxis[1]: 50-200 mg/day in the South African Medicines Formulary[172]. Recommendations are contradictory in the South African TB management guidelines: 10-25, 25 and 100 mg/day are cited in three separate instances within the same document[24]. The basis for the increased dose approach is not clear.

2.4.1.2.3 Pharmacokinetics and effect on B6 status

Oral pyridoxine, generally given as the hydrochloride, is readily absorbed from the gut into the systemic circulation by first-order kinetics; particular formulations can influence bioavailability, however[173]. Soon after oral ingestion, PLP levels rise and return close to baseline levels after 5-6 hours[162]. After a period of daily supplementation, PLP levels increase >10-fold and 4PA levels increase around 50-fold from baseline physiological values[154]. PLP levels do not increase in a dose-dependent fashion,

however,[154,163,173] and appear to be saturable at higher dosages (>50 mg/day) – PLP in plasma is bound to albumin and levels of the two are correlated[154,161]. Excess unbound PLP is probably hydrolysed and excreted as 4PA[154,163]. Consequently, there appears to be little benefit conferred by high-dose pyridoxine regimens in terms of B6 status.

As studies of B6 status during supplementation are limited to those performed on healthy volunteers, little is known about pyridoxine pharmacokinetics in disease states[163,173]. Furthermore, determination of pharmacokinetic parameters is particularly challenging. Parameters are influenced not only by the agent itself, but also by baseline B6 status, dietary and endogenous factors. Further complications arise because of the lack of consensus on the most representative marker of B6 status[154,173].

2.4.1.2.4 Toxicity

Pyridoxine in doses of ≥2 g/day is known to cause a sensory neuropathy characterised by neuropathic pain; impaired reflexes, vibration, light touch, pinprick and temperature sensation; ataxia; abnormal nerve conduction study findings; and sensory neuronopathy on histological examination[174]. These doses are far higher than those recommended for INH-PN prevention and treatment (see Section 2.4.1.2.2); doses up to 100 mg/day are considered safe[1]. A single case report describes aggravation of pre-existing INH-PN by pyridoxine at a dose of 150 mg/day[175]. *In vitro* neurotoxicity has been demonstrated in dorsal root ganglia and may be attributable to feedback inhibition of pyridoxal kinase[175].

2.4.1.3 Multivitamin supplementation

A RCT of multivitamin supplementation demonstrated reduced DSP in the treatment arm of the trial, which comprised both INH and HIV+INH participants[176], but the influence of the pyridoxine component (25 mg) could easily have accounted for the reduction in risk[177] whereas administration of vitamin B-complex not containing B6 failed to prevent INH-PN in TB mono-infected patients receiving INH[26]. Other research in our group points to a lack

of protective effect of B-complex supplementation in an out-patient ART cohort[112].

2.4.2 Non-pharmacological therapies

Withdrawal of the d-drug is a self-evident approach to the management of ATN[12,16]; dose reduction might also be effective[71]. Withdrawal of INH is indicated in severe cases of INH-PN, but pyridoxine supplementation is preferable in most cases[23,67].

Acupuncture has been trialled for HIV-associated DSP and found to be largely ineffective[170]; however, other complementary therapies such as reflexology, massage and meditation were reported to offer relief in a large multi-centre survey of HIV-associated DSP self-management. Self-care practices such as taking a hot bath, staying off feet, exercise and rubbing were also effective for some[167]. Lack of effective therapies might cause individuals to turn to alternative methods of pain control, some of which are maladaptive – up to 30% reported use of alcohol and/or illicit substances in the above-mentioned survey; in particular, more severe pain was associated with increased use of "hard" drugs[168]. The contribution of psychological factors, such as depression or pain catastrophising, to the perception of and coping mechanisms associated with neuropathic pain are under-estimated. These factors should be addressed appropriately and holistically[21,167].

2.4.3 Prevention

Pyridoxine is effective for the prophylaxis of INH-PN (see Section 2.4.1.2.2) but prevention strategies for HIV-associated DSP are limited. There is some evidence that earlier initiation of ART (at $CD4^+$ T cell counts >350 cells/μl) might be protective[13]. Identification of risk factors for DSP and the avoidance of d-drugs in patients at risk is a strategy that has received attention in the literature[12,54,55]. Avoidance of d-drugs is obviously dependent on the availability of alternatives – while d4T is being phased out in South Africa[178], the same is not true for other resource-limited settings[22].

3 Methods

Study design and methodology.

3.1 Ethics approval

The study was approved by the University of Cape Town Human Research Ethics Committee (REC REF: 079/2010).

3.2 Study design

The study design is that of a prospective analytical non-interventional cohort with a baseline cross-sectional component. The primary outcomes were baseline (prevalent) and incident or worsening DSP. The primary exposures of interest were plasma PLP deficiency and a slow predicted NAT2 acetylation phenotype.

3.2.1 Study site

The study was undertaken at DP Marais SANTA TB Hospital (DPM), a public sector hospital located in Retreat, Cape Town, South Africa.

3.2.1.1 Site access

Site access was granted by the Division of District Health Services and Programmes, Provincial Government of the Western Cape in February 2010 (2010 RP 27).

3.2.1.2 About DPM

DPM is a 260-bedded hospital which, in conjunction with Brooklyn Chest Hospital in Ysterplaat, Cape Town, provides inpatient services for adult TB patients referred from all primary, secondary and tertiary facilities in the greater Cape Town region. DPM is primarily a male hospital, while Brooklyn Chest caters for female patients; however, there are both male and female wards at each – the male-to-female bed ratio at DPM is 4.2:1. According to hospital records, the bed occupancy rates average 82.4%, with approximately 20 admissions per week. The average admission period is two months, but the range is wide – patients can be admitted for short (several days), or

extended stays (several months). The approximate mortality rate at DPM is 5-8 patients/month.

National guidelines[24] inform the management of patients at DPM. Anti-TB regimen 1 is prescribed for new TB cases while regimen 2 is for retreatment cases (**Table 3-1**). Patients receive fixed dose combinations of the oral agents according to weight; specifically, patients receive 4-6 mg/kg of INH daily, irrespective of regimen or phase of treatment. At the time of admission to DPM, the majority of patients will have already initiated anti-TB therapy at the referring facility. While not stipulated in national guidelines[24] (see Section 2.4.1.2.1), supplemental prophylactic pyridoxine-HCl is administered to all patients admitted to DPM at a dosage of at least 25 mg/day, in addition to two vitamin B-complex tablets, each containing 0.5 mg pyridoxine. The usual practice at DPM is to prescribe 25 mg/day on admission, but if DSP is diagnosed, the dose is boosted to 50 or 100 mg/day. Amitriptyline is commonly prescribed in escalating doses for symptomatic relief. All morning medications, including anti-TB therapy and pyridoxine, are dispensed daily at 08:00.

Table 3-1 Department of Health National TB Guidelines anti-TB regimens.

	Intensive phase	Continuation phase
Regimen 1	2 months: Rifampicin Isoniazid Pyrazinamide Ethambutol	4 months: Rifampicin Isoniazid
Regimen 2	3 months: Rifampicin Isoniazid Pyrazinamide Ethambutol 2 months: Streptomycin	5 months: Rifampicin Isoniazid Ethambutol

Adapted from Department of Health[24].

HIV-infected patients not on cART at the time of their admission to DPM are assessed for eligibility for treatment according to national guidelines[178], and are worked up in a separately run onsite ART clinic. Prior to May 2010 (during the study period), the first-line cART regimen NRTI backbone was either d4T

or AZT; in May 2010 the national guidelines switched d4T to tenofovir (TDF) as the preferred first-line NRTI backbone agent (but d4T could still be prescribed if TDF and AZT were contraindicated, and could be continued in those stable on therapy) (**Table 3-2**).

Table 3-2 New South African Antiretroviral Treatment Guidelines regimens.

	NRTI "backbone"	Second NRTI	NNRTI/PI
First-line regimen	Tenofovir (TDF) or Zidovudine (AZT) or Stavudine (d4T)	Lamivudine (3TC) or Emtricitabine (FTC)	Efavirenz (EFV) or Nevirapine (NVP)
Second-line regimen	Zidovudine (AZT) or Tenofovir (TDF)	Lamivudine (3TC) or Emtricitabine (FTC)	Lopinavir + Ritonavir (LPV/r)

NRTI=nucleoside/nucleotide reverse transcriptase inhibitor, NNRTI=non-nucleoside reverse transcriptase inhibitor, PI=protease inhibitor
Adapted from Department of Health[178].

Similar to many public sector institutions in South Africa, DPM is a resource-constrained environment – for example, with one part-time doctor and three full-time doctors during the study period, the patient-doctor ratio was around 60:1. The hospital also has no x-ray facilities, no on-site laboratory and a limited dispensary.

3.2.1.3 Patient population

Patients are referred to DPM for a variety of reasons including, but not limited to, a requirement for continuing inpatient care after stabilisation on anti-TB therapy; significant co-morbidities or disability; drug resistant TB; adverse drug reactions necessitating monitoring and drug re-challenge; a poor compliance history; indigence or other social issues; substance abuse; mental illness or other cognitive impairment; and poor clinic access (particularly for patients on regimen 2 who require daily intramuscular streptomycin injections). The result is a heterogeneous inpatient population comprising both relatively well and unwell patients. The former are admitted for mostly practical reasons and are ambulant, while the latter are largely ill, but stable, patients with co-morbidities – acutely ill patients cannot be accommodated at DPM. Around 70% of admissions to DPM are HIV co-infected, and represent a spectrum of degree of immune suppression, and current or previous exposure to ART (see also description of the study sample in Section 4.2.1).

3.2.2 Selection criteria and sampling methods

DPM inpatients satisfying the following criteria were eligible for enrolment in the study:

Inclusion criteria

1. Age ≥ 18;
2. HIV/TB co-infection; and
3. The patient understood the purpose and requirements of the study and provided written informed consent.

Exclusion criteria

1. Cognitive impairment which was felt to preclude informed consent or cooperation with the study protocol;
2. Signs and symptoms suggestive of either a radiculopathy or myelopathy, or other focal neurological signs, that, in the opinion of the investigator, complicated the diagnosis of DSP;
3. Spinal or CNS infection – these carry an increased risk for potentially confounding focal neurology and/or cognitive impairment;
4. Patient was deemed generally too ill to participate in the study or to reliably complete study follow-up;
5. The use of non-standard anti-TB therapy regimens, such as those for post-drug toxicity re-challenge or multi-drug resistant TB – because of variation in treatment exposures and potential confounding;
6. Diabetes mellitus – because of the high risk of potentially confounding peripheral neuropathy;
7. Pregnancy – because of potentially confounding vitamin B6 deficiency;
8. Inability to obtain consent because the patient was not aware of or in denial about their HIV status; and
9. Inability to assess limbs symmetrically – for example, one or more limbs immobilised or amputated.

The following criteria were also applied during the initial pilot period, before the study design was modified (see Section 3.2.5):

1. ART-naïve or no ART exposure within the preceding three months; and
2. Qualified for ART according to national guidelines (at the time, $CD4^+$ T cell count <200 cells/μl).

During the initial pilot period (see Section 3.2.5), sampling was convenience; thereafter, sampling was sequential (consecutive eligible admissions).

3.2.3 Case definitions and outcomes

DSP was defined as the presence of ≥1 neuropathic symptom and ≥1 neuropathic sign. No DSP or DSP-free was defined in all participants not satisfying the above criteria, including those classified with ADSP (symptom-free and with ≥1 neuropathic sign).

Incident DSP was defined in either those participants who were DSP-free at baseline and subsequently developed DSP at follow-up (thus including those with ADSP at baseline who developed symptoms at follow-up). Worsening DSP was defined in participants who had DSP at baseline and subsequently demonstrated a 2-point increase in the NRS for pain and/or paraesthesia, or a 2-point increase in the composite 20-point TNS score (for a description of the TNS see Sections 2.2.4.3 and 3.3.6). Longitudinal outcomes are tabulated in **Table 3-3**.

Table 3-3 Incident and worsening DSP definitions.

Baseline	Follow-up	
	Incident DSP	Worsening DSP
DSP-free	New symptoms and signs	
Asymptomatic DSP	New symptoms	
Symptoms only	New signs	
(Symptomatic) DSP		2-point increase in NRS for pain and/or paraesthesia, or 2-point increase in TNS score*

DSP=distal sensory polyneuropathy, NRS=numerical rating scale, TNS=Total Neuropathy Score
*See Section 3.3.6 for descriptions of symptom and TNS scoring.

DSP severity was gauged by the composite 20-point TNS score. Higher scores denoted more severe DSP.

Participant recall of the temporal relationship between neuropathic symptom onset and the onset of TB symptoms, the initiation of anti-TB therapy and the initiation of cART if he or she was receiving cART, guided the assignment of a baseline aetiological diagnosis: HIV-DSP, ATN or INH-PN. Neuropathic symptom onset concurrent with TB symptom onset was further labelled as possible "TB-DSP" (**Table 3-4**).

Table 3-4 Assignment of aetiological diagnosis based on participant recall of relation of neuropathic symptom onset to TB symptoms, anti-TB therapy and ART.

Neuropathic symptom onset*		Diagnosis
Before TB symptoms	Before ART / not on ART	HIV-DSP
	After ART	ATN
Concurrent with TB symptoms but before anti-TB therapy	Before ART / not on ART	HIV-DSP (or "TB-DSP")
	After ART	ATN
After anti-TB therapy	Before ART / not on ART	INH-PN
	After ART	Indeterminate

DSP=distal sensory polyneuropathy, ART=antiretroviral therapy, ATN=antiretroviral toxic neuropathy, INH-PN=isoniazid-induced peripheral neuropathy
*Based on participant recall.

3.2.4 Study schedule and censorship

Participants were assessed at baseline shortly after admission to DPM and then four-weekly for the period of admission (**Table 3-5**).

Study censorship occurred on patient discharge or transfer from DPM (including self-discharge); withdrawal of consent (opting out); the development of drug toxicity and acute illness preventing continuation with the study; or at the end of the study period (17 August 2011). Transferred patients who later returned to DPM continued to be followed-up monthly if possible, even if they had missed one or more visits.

Table 3-5 Study schema.

Evaluation	Baseline	Longitudinal follow-up*
Informed consent	X	
Documentation of HIV status	X	
Medical and social history	X	
Medication review (anti-TB therapy, ART, pyridoxine or other)	X	X
Neuropathy assessments		
BPNS	X	X
TNS	X	X
Laboratory assessments		
NAT2 genotype (9 ml EDTA)	X	
PLP, 4PA (9 ml EDTA)	X	X

ART=antiretroviral therapy, BPNS=Brief Peripheral Neuropathy Screen, TNS=Total Neuropathy Score, *NAT2=n-acetyltransferase 2,* PLP=pyridoxal 5'-phosphate, 4PA=4-pyridoxic acid
*Longitudinal follow-up performed four-weekly for duration of admission.

3.2.5 Pilot study

An initial pilot period ran from 1 March - 26 April 2010. In the original study protocol, it was envisaged that participants would be followed from just prior to the initiation of cART and for a period of at least two months thereafter; the emphasis was on longitudinally assessing the impact of ART on vitamin B6 status and the risk for DSP (this design is employed by another study in our group). However, during the pilot period, it was found that in many cases the period of admission was far shorter than anticipated. It also became apparent that only a minority of patients were ART-naïve and also eligible for cART (according to the more restrictive national guidelines in place at the time) and in those patients who were eligible for cART, there was a significant lag between admission and cART initiation. The potential for long-term follow-up was then further reduced. Consequently, a decision was taken by our group to relax the study criteria to include all HIV-infected admissions regardless of ART treatment status (see Section 3.2.2). This would improve enrolment rates and also provide a baseline comparator. Pilot participants were included as part of the final sample but were not included in descriptions and comparisons of baseline ART use. They were, however, included in the multivariate analysis.

3.3 Study procedures

3.3.1 Field visits

Field visits to DPM took place bi-weekly (Mondays and Wednesdays). During the morning, potential and enrolled study participants were identified and located. Two lists were compiled and provided by DPM administrative staff for this purpose: a recent admissions list and a discharge list. Folders of new admissions were reviewed for eligibility. Participants already enrolled and due for imminent discharge were prioritised for follow-up assessment if this had not occurred in the preceding two weeks. Study assessments were begun at 13:00 and were done consecutively.

3.3.2 Facilities

Study procedures, including taking of informed consent, clinical assessments and blood sampling, were conducted in a private consultation room allocated specifically for study purposes during the afternoons of field visit days.

3.3.3 Personnel

All field visits and nearly all neurological assessments were performed personally. I was accompanied on most visits to DPM by a registered nurse who assisted with translation, informed consent, folder review, participant co-ordination and blood sampling.

3.3.4 Informed consent

All subjects provided written informed consent and retained a patient information sheet (Appendix 1). The information sheet was not available in translated versions; however, the consent process was performed in the preferred language of the subject (English, Afrikaans or IsiXhosa) by a fluent speaker. Consent was provided for both the clinical and investigational aspects of the study – permission was granted or denied for the *NAT2* genetic analysis specifically, as well as for storage of samples for future use.

3.3.5 General history and folder review

The following data were documented based on history provided by the participant and confirmed where necessary by folder review: general medical history including previous TB episodes; duration of current TB episode; site of TB infection; anti-TB regimen administered; HIV status and period of infection; WHO clinical stage; previous ART exposure and/or current cART regimen; current and previous pyridoxine dose and period of supplementation; and other current medication. If a diagnosis of DSP was recorded in the patient folder, this was also documented. A single question alcohol screen[179] was used to identify problem drinking. Height and weight measurements were obtained. Self-assigned race category was obtained from hospital records.

Results of the following basic chemistry, haematology and serology laboratory investigations, provided by the National Health Laboratory Services (NHLS), were obtained by folder review: serum urea, creatinine, alanine transferase and aspartate transferase; white cell count, haemoglobin, mean red cell volume, platelet count and $CD4^+$ T cell count; and hepatitis B surface antigen. Results of investigations confirming TB infection (direct observation of acid fast bacilli, culture, polymerase chain reaction, fluid adenosine deaminase or radiological confirmation) were also obtained. Where results were unavailable an attempt was made to locate them using the NHLS website.

Data were recorded on a data collection form (Appendix 2).

3.3.6 DSP assessment

DSP was assessed using both the BPNS and the modified clinical TNS[11] (see Section 2.2.4.3), complemented by additional questioning.

3.3.6.1 Neuropathic symptoms

To assess neuropathic symptoms, participants were shown three 11-point visual NRSs (also translated into isiXhosa) for each of the modalities of pain, paraesthesia and numbness, as per the BPNS. Participants selected a rating from 0 (no pain) to 10 (worst pain) for each. The single highest NRS score of the three modalities was then used to establish a sensory severity grade

(grade 0 for no symptoms; 1 for a highest score of 1-3; 2 for 4-6; 3 for 7-8; and 4 for 9-10). In the TNS, sensory symptoms in all modalities are assessed as a single entity and scored by anatomical extent rather than severity. A higher score is obtained for more proximal involvement (0 for no symptoms; 1 for soles/toe; 2 for ankle; 3 for knee; and 4 for more proximal involvement). In addition to the above, the presence or absence of lower limb cramps was established.

An effort was made to characterise symptoms as neuropathic in nature; that is, distal-to-proximal, symmetrical and not related to other pathologies (spinal, joint, musculoskeletal or dermatological). If the pattern of symptoms was obviously non-neuropathic in nature this was noted on the data collection form and a score of 0 was given.

If symptoms were absent at the time of the baseline assessment, an enquiry was made as to whether any symptoms had been experienced in the past (yes or no). At baseline, participants were asked to recall the onset of symptoms (if present) relative to the onset of TB symptoms and the initiation of anti-TB therapy (before TB symptoms; concurrent with TB symptoms but before anti-TB therapy; after anti-TB therapy; or unsure). At baseline and follow-up, prevalent or incident symptoms were compared to the onset of cART initiation (before, after or unsure). At follow-up, a 5-point patient global impression of change (PGIC) was used to assess subjective change in symptom status if symptoms had been present at the previous visit.

3.3.6.2 Neuropathic signs

For the examination portion of the assessment, subjects were seated facing the examiner with their lower limbs exposed and hanging over the edge of an examination couch. All procedures were explained clearly to the patient beforehand. Vibration sense was assessed at the distal interphalangeal joint of each first toe by the firm application of a vibrating 128 Hz tuning fork. In the BPNS, the vibratory thresholds are recorded for each toe and a score is obtained (0 for >10s; 1 for 6-10s; 2 for <6s; and 0 if not perceived). In the

TNS, the extent of impaired vibratory thresholds (<10s) is recorded in a similar fashion to that for symptoms (see above).

Tendon reflexes were assessed using a long-handled patellar hammer with a weighted circular rubberised head. Ankle reflexes were elicited by first dorsiflexing the foot with one hand and then striking the Achilles tendon with the hammer. While dorsiflexing a finger was placed on the tibialis anterior tendon to ensure the subject was not flexing of his or her own accord. Clonus was assessed by rapid dorsiflexion of the foot; more than two beats was considered abnormal. The ankle reflex response was assessed using that of the knees as a reference. In the BPNS, only ankle reflexes are considered (4 for absent; 3 for reduced; 2 for normal; 1 for hyperactive; and 0 for clonus). In the TNS, all reflexes are considered (0 for all normal; 1 for ankle reflexes reduced; 2 for ankle reflexes absent; 3 for ankle reflexes absent and others reduced; and 4 for all reflexes absent).

Pinprick sensation and power were assessed as part of the TNS. Impairment of pinprick sensation was assessed anatomically, again scored as for symptoms (see above), while toe/ankle dorsiflexion was assessed using the Medical Research Council (MRC) grading system (0-4). In addition, proprioception at the big toe was assessed (normal, reduced or absent).

Neuropathy findings were recorded on a neuropathy capture form (Appendix 3).

3.3.7 Blood sampling and sample handling

Peripheral venous blood was drawn with 22 gauge needles by standard techniques and utilising the Vacutainer system (BD, Franklin Lakes, NJ, USA). If venous access was difficult, no more than two attempts at venepuncture were made. One 9 ml clot-activated sample (for serum) and one EDTA sample (for buffy coat and plasma) were obtained at each visit (**Table 3-5**). The samples were placed immediately within a cool box containing frozen ice packs. The samples were centrifuged at 4 G within 1-4 hours and stored at -80ºC.

The number of hours that had elapsed since the morning dosing of pyridoxine supplementation (08:00) was recorded. Samples were obtained a minimum of five hours post-dosing; plasma PLP levels approach pre-trough levels after 4 6 hours[162].

3.4 Plasma vitamin B6 HPLC

Plasma PLP and 4PA were determined by HPLC at the Centers for Disease Control and Prevention, Atlanta, GA, USA. Samples for a pilot analysis were selected sequentially: the first 25 patients with any follow-up data were analysed. Preparation of the samples was as follows: plasma was fractioned from whole blood by centrifugation within 1-4 hours of sample acquisition; exposure to light was minimised and the samples were kept cool in the interim period. Storage was at -80℃ until cold-chain air shipp ing to Atlanta. HPLC was performed according to the method of Rybak et al.[42,180]. Vitamin B6 deficiency was defined as <30 nmol/l[153].

3.5 NAT2 molecular methods

NAT2 genetic variation was assessed by both genotyping and sequencing, and NAT2 acetylation phenotype predicted in a subset of the cohort (all participants with any longitudinal follow-up data).

3.5.1 Genotyping

Genotyping was performed utilising a combination of polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) analysis and allele-specific amplification, as adapted from the method of Doll et al.[181]. Genotype was determined at the following positions: 191, 282, 341, 434, 481, 590, 803, 845 and 857.

3.5.1.1 *NAT2* amplification by polymerase chain reaction

Deoxyribonucleic acid (DNA) was extracted from buffy coat isolated from peripheral blood using the QIAamp Mini Kit (Qiagen, Hilden, Germany) according to the instructions of the manufacturer. Each DNA sample was titrated to 50 ng/µl if >50 ng/µl. The *NAT2* gene was then amplified by

optimised PCR in a 100 µl mixture containing ≤150 ng DNA; 0.04 µmol mixed dideoxynucleotides (dNTPs); 0.01 µmol $MgCl_2$; and 2.5 units (u) *Taq* polymerase in corresponding reaction buffer (Promega, Madison, WI, USA) and distilled water using the *NAT2*-specific primers, 5'-ATGGACATTGAAGCATATTTT-3' (forward) and 5'-AAGGGTTTATTTTG-TTCCTTAT-3' (reverse) (Integrated DNA Technologies, Coralville, IA, USA), under thermocycling conditions summarised in **Table 3-6**. The resulting predicted 895 bp fragment was then electrophoresed in an ethidium bromide-containing agarose gel and visualised under ultraviolet light.

Table 3-6 Cycling conditions for *NAT2* PCR, allele-specific PCR and cycle sequencing.

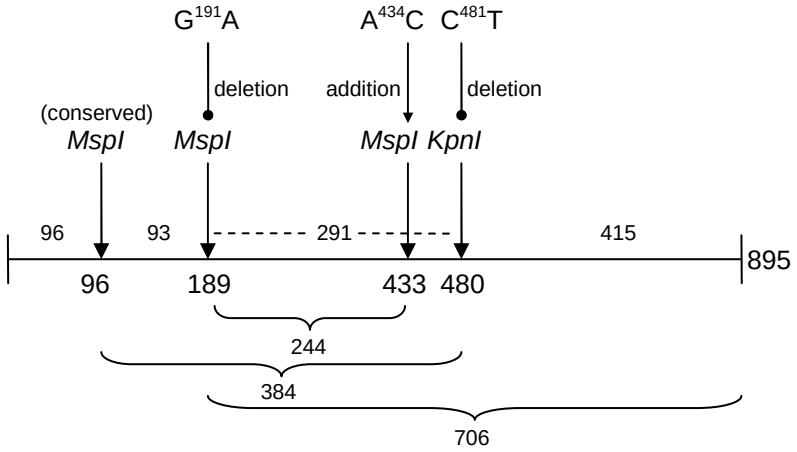
	NAT2 PCR			Allele-specific PCR			Cycle sequencing		
PCR step	Temperature*	Duration		Temperature*	Duration		Temperature*	Duration	
Activation	94º	3 min		94º	3 min		98º	5 min	
Denaturation	94º	1 min	30 cycles	94º	1 min	30 cycles	96º	30 sec	30 cycles
Annealing	55º	1 min		58º	1 min		50º	15 sec	
Extension	72º	1 min		72º	1 min		60º	4 min	
Final extension	72º	5 min		72º	5 min		-	-	

PCR=polymerase chain reaction.
*Temperatures are in degrees Celsius.

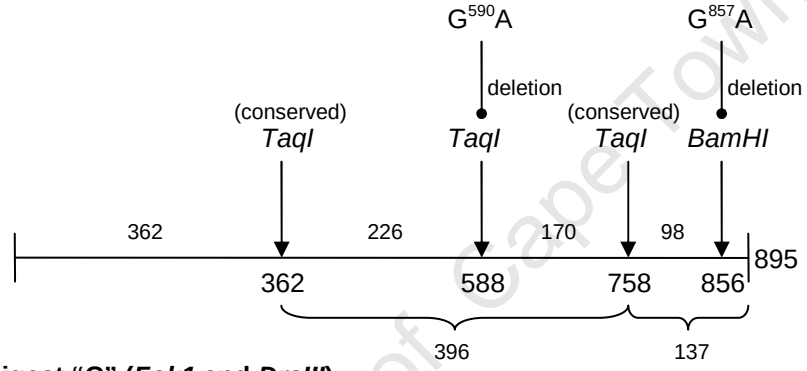
3.5.1.2 Restriction fragment length polymorphism analysis

Seven restriction enzymes in four separate reactions, “A”, “B”, “C” and ”D”, were used to identify the presence of eight different SNPs in the *NAT2* gene: $G^{191}A$, $C^{282}T$, $A^{434}C$, $C^{481}T$, $G^{590}A$, $A^{803}G$, $A^{845}C$ and $G^{857}A$. The restriction enzymes used in each reaction, along with their recognition and cleavage sites, are listed in **Table 3-7**. A schematic representation of the cleavage of the 895 bp *NAT2* fragment, as predicted by RestrictionMapper version 3 (available from: http://www.restrictionmapper.org/), is presented in **Figure 3-1**. Each 40 µl enzyme digestion reaction contained 20 µl PCR products and 10 u of each restriction enzyme in corresponding reaction buffer (Fermentas, Vilnius, Lithuania) and distilled water, and was incubated as per **Table 3-6**. Digestion products were applied to a 2-3% ethidium bromide-containing agarose gel and electrophoresed at 80-100 V for 1-2.5 hours, visualised and photographed.

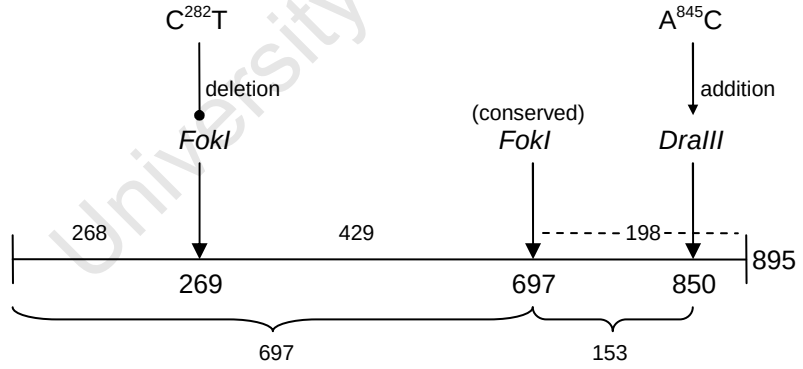
Digest "A" (*MspI* and *KpnI*)



Digest "B" (*TaqI* and *BamHI*)



Digest "C" (*Fok1* and *DraIII*)



Digest "D" (*DdeI*)

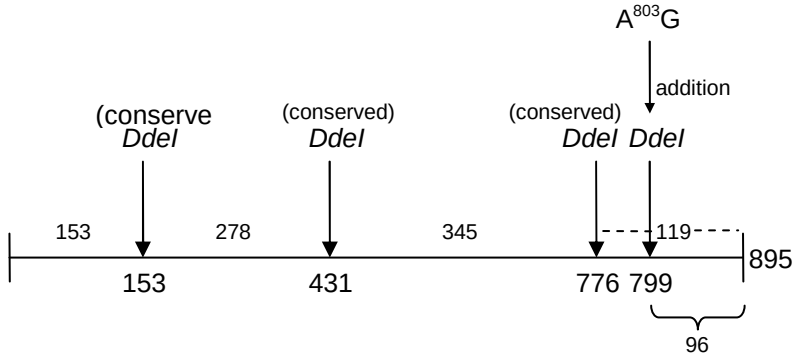


Figure 3-1 Schema of the predicted cleavage of the *NAT2* fragment in each restriction enzyme digestion "A", "B", "C" and "D".

Table 3-7 Restriction enzyme digestion mixtures, recognition and cleavage sites and incubation conditions.

Digestion	Enzymes*	Recognition site**	Temperature (C)	Duration
"A"	*MspI* *KpnI*	5'-C^CGG-3' (2) 5'-GTAC^C-3' (4)	37°	3 h
"B"	*TaqI* *BamHI*	5'-T^CGA-3' (2) 5'-G^GATCC-3' (4)	37° 65°	2 h
"C"	*BseGI* (*FokI*) *AdeI* (*DraIII*)	5'-GGATG$(N)_9$^-3' (4) 5'-CACNNN^GTG-3' (-3)	37°	3 h
"D"	*HpyF3I* (*DdeI*)	5'-C^TNAG-3' (3)	37°	3 h

**BseGI* corresponds to *FokI*, *AdeI* to *DraIII* and *HpyF3I* to *DdeI*.
**The 5'-3' recognition sequence only is presented; the point of cleavage is represented by a caret. Overhang on the complementary strand is represented in parentheses as the number of base pairs difference; for *AdeI*/*DraIII* the cleaved 3'-5' strand has a 3 bp overhang.

3.5.1.3 Allele-specific amplification

In order to detect the presence of the $T^{341}C$ SNP, allele-specific PCR was employed. After optimisation for temperature and $MgCl_2$ concentration, a 25 µl reaction mixture was set up containing ≤100 ng genomic DNA; 0.01 µmol mixed dNTPs; 0.04 µmol $MgCl_2$ and 1 u *Taq* polymerase in corresponding reaction buffer (Promega, Madison, WI, USA) and distilled water, and run according to cycling conditions in **Table 3-6**. In two separate reactions, the forward primer, 5'-CTCCTGCAGGTGACCAT-3', was used to selectively amplify wild-type *NAT2* at the 341 locus and 5'-CTCCTGCAGGTGACCAC-3' was used to amplify the C-substitution. The reverse primer used in both reactions was 5'-GGAGACGTCTGCAGGTATG-3'. All primers utilised in the allele-specific PCR were manufactured by Inqaba Biotec (Cape Town, South Africa). Each reaction was loaded separately onto a 1.5% ethidium bromide-containing agarose gel and electrophoresed at 160 V for 45 min before visualisation.

3.5.2 Sequencing

Confirmation of genotype by sequencing was performed in a subset of samples. Cycle sequencing of 10 µl reactions containing 2 µl PCR products; 2 µl dye termination mix and 2 µl corresponding buffer (Applied Biosystems, Foster City, CA, USA); and 3 µl distilled water was done according to cycling conditions in **Table 3-6** and using, in separate reactions, either or both

forward and reverse *NAT2* primers as per Section 3.5.1.1. After ethanol precipitation, the samples were capillary electrophoresed on an ABI PRISM 3130X genetic analyser (Applied Biosystems, Foster City, CA, USA) and an electropherogram was generated.

Two computer programs, BioEdit Sequence Alignment Editor version 7.0.9.0[182] and ChromasPro version 1.5 (Technelysium Pty Ltd, Tewantin, QLD, Australia) were used to visually examine the electropherogram and confirm the corresponding nucleotide sequence before aligning both forward and reverse sequences with the reference *NAT2*4* sequence (GenBank: X14672.1).

3.5.3 Genetic analysis and phenotype prediction

SNP frequencies were evaluated for Hardy-Weinberg equilibrium using the χ^2 test for goodness of fit to detect differences between observed and predicted frequencies of homozygous and heterozygous SNPs. The level of significance was 0.05 (df=1).

A diplotype with nine positions (191, 282, 341, 434, 481, 590, 803, 845 and 857) was generated based on: 0=two wild-type alleles, 1=heterozygous for the SNP and 2=homozygous for the SNP. Probable *NAT2* haplotypes and frequencies were then predicted using computational haplotyping: diplotypes were entered into PHASE version 2.1.1[183,184] and initial settings set at 1 000 iterations, 500 burn-in iterations and a thinning interval of 1, as described by Agúndez et al.[146]. As this resulted in inconsistent total numbers and frequencies of predicted haplotypes, burn-in iterations were increased to 10 000, and iterations increased stepwise from 10 000 through 20 000, 100 000 and 500 000 until haplotype frequencies were consistent within 0.1% among eight independent runs, each run based on a random number seed obtained from RANDOM.ORG (available from: http://www.random.org). Recombinant rate variation modelling was not specified, and the default model was used. Predicted haplotypes were then matched to known haplotypes in Human *NAT2* alleles (Haplotypes) (available from: http://n-acetyltransferasenomenclature.louisville.edu/)[139].

Phenotype was predicted based on the number of homozygous or heterozygous slow alleles: $G^{191}A$, $T^{341}C$, $A^{434}C$, $G^{590}A$, $A^{845}C$ and $G^{857}A$ (**Table 3-8**). In the case of heterozygosity for two or more slow alleles, the ambiguity in the prediction of phenotype was as a result of the inability to determine the phase of the slow alleles (i.e. if located on the same chromosome the predicted phenotype would be intermediate; if located on opposite chromosomes, slow). The phase of ambiguous genotypes was informed by the reconstructed haplotypes generated by PHASE.

Table 3-8 Prediction of phenotype based on number of homozygous and heterozygous slow alleles.

Homozygous slow alleles*	Heterozygous slow alleles*	Predicted phenotype
0	0	Fast
0	1	Intermediate
0	≥2	Intermediate or slow (ambiguous)
≥1	Any number	Slow

*Slow alleles were $G^{191}A$, $T^{341}C$, $A^{434}C$, $G^{590}A$, $A^{845}C$ and $G^{857}A$.

To assess genotypic associations with outcomes, binary variables were generated. For SNPs: homozygous vs. heterozygous/wild type alleles, and for phenotypes: slow vs. intermediate/fast. Haplotype frequencies were compared between those with and without baseline DSP, and between race groups, using the PHASE case-control function.

3.6 Data management and analysis

3.6.1 Data capture and management

Data were captured personally and entered into an Access 2007 relational database (Microsoft Corporation, Redmond, WA, USA). The database was designed in two parts: a baseline cross-sectional table and a stacked longitudinal table. Queries were built using the Structured Query Language to extract and recode data from the database while preserving the integrity of the original dataset. The database was password-protected and contained no identifiable data other than patient initials and date of birth. Data were imported directly and dynamically into the statistical software using Open Database Connectivity.

3.6.2 Statistical methods

Stata version 11 (College Station, TX, USA) was used for statistical analysis. STATA is one of only a few widely available packages capable of performing a generalised linear models analysis for estimating prevalence ratios (see below). Graphical representations, other than those generated by Stata for the survival analysis, were drawn by GraphPad Prism version 5.04 (La Jolla, CA, USA). Probability (*p*) values were considered significant at <0.05. All statistical analyses were supervised by a statistician, Dr Henri Carrara, School of Public Health and Medicine, University of Cape Town.

3.6.2.1 Baseline (cross-sectional) data

Descriptive statistics were used to define the study sample: binary data were described by frequencies and proportions; the mean and standard deviation (SD) were calculated for continuous data that were established as normally distributed on Shapiro-Wilk testing ($p \geq 0.05$), or after appropriate transformation. The median and first to third quartiles (Q1-Q3) were calculated for ordinal or non-normally distributed continuous data. However, the mean and SD were used to describe neuropathy scores. Baseline characteristics were tested for association with baseline DSP by the use of the χ^2 test, rank-sum test and Student's *t*-test, as appropriate. The prevalence ratio (PR) and 95%CI was then estimated by generalised linear models using Stata's `glm` command (a Gaussian distribution was specified) with DSP as the dependent variable. All crude ratios were adjusted for age and gender. Multivariate analysis was performed based on $p<0.2$ on univariate analysis. The strength and significance of the correlation between numerical variables was established by Pearson and Spearman rank-sum testing: for normally distributed continuous variables the former was utilised, and for one or two ordinal or non-normally distributed continuous variables, the latter. GraphPad Prism was then used to estimate and plot the function and 95%CI of the correlation by linear regression.

3.6.2.2 Follow-up (longitudinal) data

The Student's *t*-test for paired samples and Wilcoxon rank-sum test for repeated measures were used to compare PLP and 4PA levels at baseline

and follow-up. Non-parametric trend estimation using Stata's `nptrend` command was employed to assess the significance of increasing or decreasing trends in the values of continuous variables over multiple time points. DSP incidence and rate of worsening with corresponding 95%CIs were calculated using Stata's `stptime` command and the denominator adjusted to 100 person-months. A Kaplan-Meir survival analysis described the probabilities of remaining DSP-free, or free of worsening DSP, through the course of the study. A "left-shifted" survival analysis was also run using historical data collected from participants and folders – the date of initiation of anti-TB therapy was used as the origin of the analysis, and participant report of symptom onset relative to this "virtual" time point informed failure status. In other words, if symptom onset was after anti-TB therapy initiation, the participant would fail at the baseline assessment. The Cox proportional hazards model compared the hazards of incident/worsening DSP conferred by fixed baseline covariates, and a hazard ratio (HR) plus 95%CI was calculated. Again, all crude ratios were adjusted for age and gender, and multivariate analysis was performed based on $p<0.2$ on univariate analysis.

3.6.2.3 Sample size

Based on 170 potentially eligible patients at DPM in a 12-month period, and expecting a 60% (+/- 5%) DSP frequency (based on Maritz et al.[11]), 117 patients were needed for a 95% confidence level.

4 Results

The study findings are presented in terms of two analyses: a cross-sectional analysis at baseline and a longitudinal analysis. Results of the PLP/4PA HPLC and *NAT2* genetic analysis are presented in detail separately.

4.1 Sample

The study sample comprised 116 participants. During the period 1-24 March 2010, prior to systematic screening of admissions, 14 eligible patients referred by DPM medical staff provided informed consent and enrolled into the study. Between 25 March 2010 and 11 April 2011[*], 881 admissions were screened, of which a further 109 patients met eligibility criteria, provided informed consent and enrolled into the study (**Figure 4-1**). Of the 160 patients who were approached to take part in the study, 36 (23.0%) declined to participate and 15 (9.0%) were unable to provide consent because they were either unaware of their HIV infection status or because they did not understand the purpose and procedure of the study. Twenty-one pilot participants were included in the final sample.

After enrolment, six participants were excluded because of the uncovering of pre-existing focal neurological signs or neurological disease. An additional participant (T095) who, at baseline, had distal symmetrical sensory symptoms and signs subsequently developed a motor neuropathy; nerve conduction studies confirmed demyelinating sensorimotor polyneuropathy and he was excluded.

Eighty-one participants (70.0%) had one or more follow-up assessments; 55 (47.0%) had two follow-up assessments (i.e. up to and including the eight week assessment), the minimum follow-up period anticipated at the start of the study. The mean attrition rate was 43.3% per visit. For those participants with any follow-up, the median duration of participation in the study was 56

[*]There was a one month break in recruitment from 18/11/10-14/12/10 due to an injury.

days (1st quartile [Q1]-3rd quartile [Q3] 29-63). Reasons for loss-to-follow-up were varied (**Figure 4-2**).

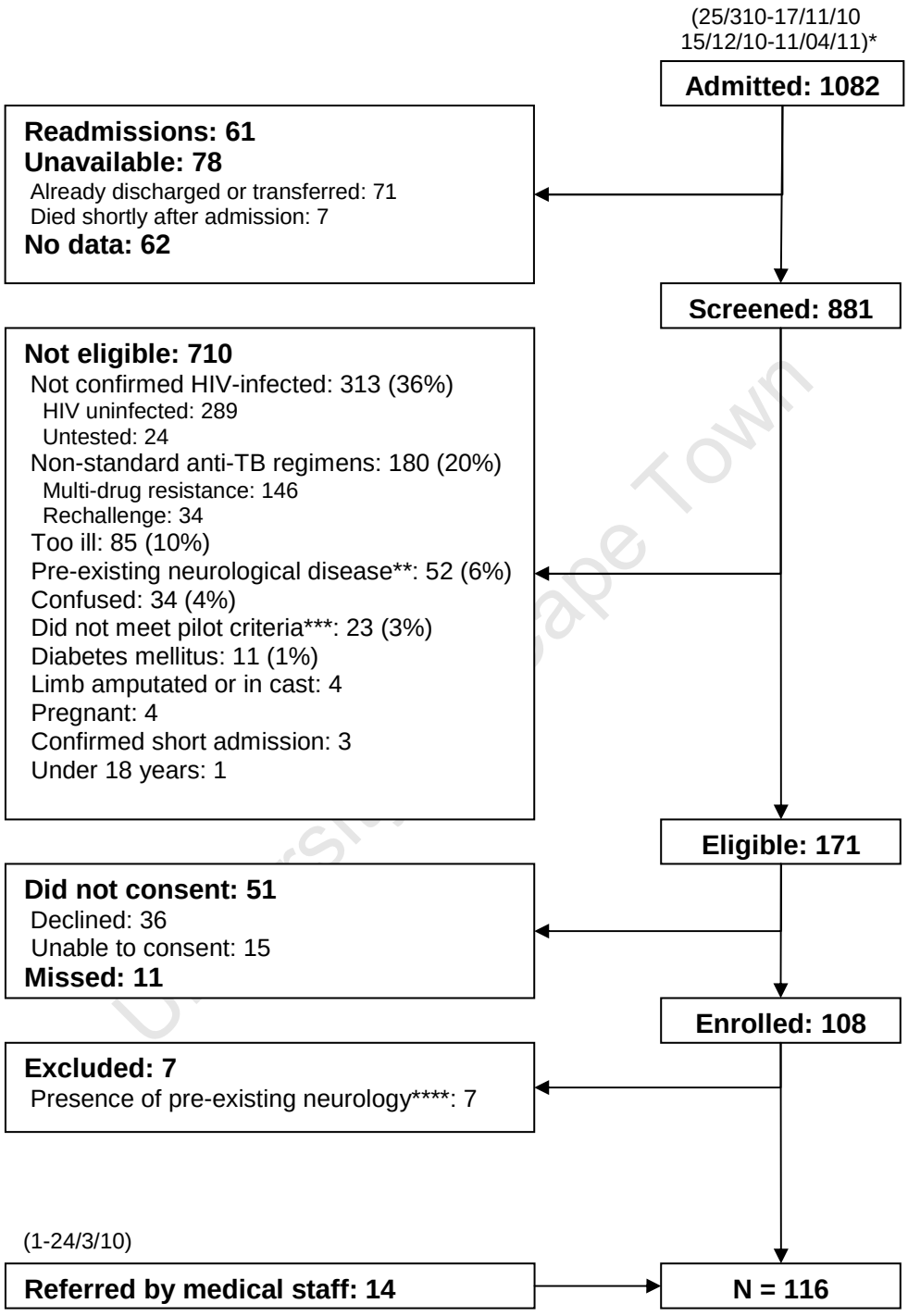


Figure 4-1 Flowchart of screening, selection and enrolment process. *Tthere was a one month break in recruitment from 18/11/10-14/12/10 due to an injury. **Based on folder review. ***See Section 3.2.5. ****Based on examination plus further folder review.

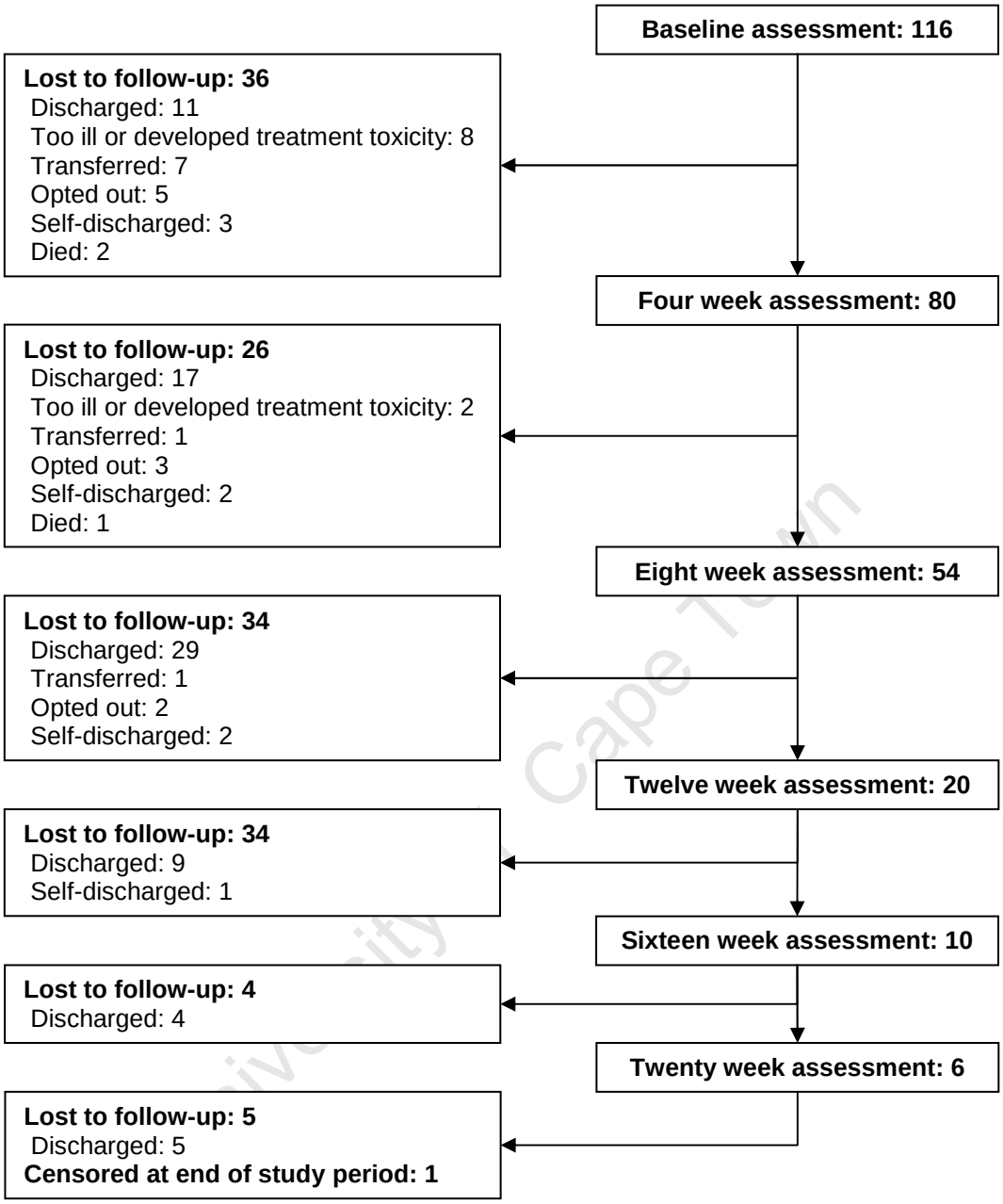


Figure 4-2 Flowchart of participant retention and attrition.

4.2 Baseline cross-sectional analysis

4.2.1 Baseline characteristics

Baseline characteristics of the study sample are summarised in **Table 4-1**. All participants had reliably confirmed TB infection, and HIV infection was documented in the clinical notes of all participants enrolled; all participants

confirmed that they were aware of their HIV status. HIV infection was present (time since diagnosis) a median of nine months (Q1-Q3 2.4-46.2), as documented in the clinical notes, or based on participant recall. The majority were either black South African (predominantly isiXhosa-speaking) (52.6%), or mixed ancestry (46.5%); one participant was black Mozambican.

Table 4-1 Baseline demographic and clinical characteristics.

Characteristic	Reference	n=116	
Age, years, mean (SD)*			35.9 (8.4)
Female, n (%)			64 (55.2)
Race, n (%)			
Black			62 (53.5)
Mixed			54 (46.5)
Weight, kg, mean (SD)*			48.7 (9.9)
Height, cm, median (Q1-Q3)		n=112	164.5 (157-170)
BMI, kg/m^2, mean (SD)*		n=112	18.3 (3.6)
Problem alcohol use, n (%)			41 (35.3)
Renal dysfunction, n (%)			8 (6.9)
TB diagnosis, n (%)			
Pulmonary			75 (64.7)
Extra-pulmonary			13 (11.2)
Disseminated			28 (24.1)
INH/weight, mg/kg, median (Q1-Q3)			5.0 (4.6-5.4)
Previous TB, n (%)			71 (61.2)
Pyridoxine dose, mg/day, n (%)			
25			88 (75.9)
≥50			28 (24.1)
WHO stage IV, n (%)			61 (52.6)
$CD4^+$, cells/μl^3, median (Q1-Q3)		n=114	101.5 (45-189)
Current cART, n (%)		n=95**	24 (25.3)
d4T backbone, n (%)			10 (10.5)
TDF backbone, n (%)			9 (9.5)
AZT backbone, n (%)			5 (5.3)
Previous cART, n (%)			28 (24.1)
cART ever, n (%)		n=95**	39 (33.6)
d4T ever, n (%)		n=95**	17 (14.7)
Creatinine, µmol/l, median (Q1-Q3)	49-104	n=107	63 (50-76)
eGFR***, ml/min, median (Q1-Q3)		n=107	92.4 (66.9-117.8)
ALT, IU/l, mean (SD)*	5-40	n=89	34.8 (49.2)
Hb, g/dl, median (Q1-Q3)*	13-17	n=104	9.3 (7.6-10.2)
WCC, x 10^9/l, median (Q1-Q3)*	4-10	n=103	6.5 (3.6-8.6)

SD=standard deviation, Q1=1st quartile, Q3=3rd quartile, BMI=body mass index, INH=isoniazid, WHO=World Health Organisation, $CD4^+$=$CD4^+$ T cell count, cART=combination antiretroviral therapy, d4T=stavudine, TDF=tenofovir, AZT=zidovudine, cART=combination antiretroviral therapy, eGFR=estimated glomerular filtration rate, ALT=alanine transaminase, Hb=haemoglobin, WCC=white cell count.
*Data deemed normal by Shapiro-Wilk testing (p≥0.05) (after transformation to inverse for age and BMI; to inverse of square root for weight and ALT; and without transformation for Hb).
**Pilot sample excluded.
***Calculated using the Cockcroft-Gault formula.

The baseline assessment was performed a median of 5 days (Q1-Q3 2-7) after admission to DPM and 16.5 days (Q1-Q3 11-39.5) after the initiation of anti-TB therapy. In the majority (90.5%), anti-TB therapy was commenced at the referral facility; the remainder initiated treatment after admission to DPM (**Figure 4-3**). All participants were receiving intensive phase anti-TB regimens at the time of the baseline assessment. Regimen 2 (see Section 3.2.1.2) was prescribed for TB retreatment in 68 (58.6%) participants; three participants with a history of previous TB did not receive regimen 2.

All participants were receiving at least 25 mg/day of supplementary pyridoxine at the time of the baseline assessment; however, 41 (39% of those who initiated treatment at a referral facility) did not recall having received pyridoxine supplementation prior to referral, and there was also no documentation of supplementation in the transfer letter of these participants. Most participants also received two tablets of vitamin B complex daily, each tablet containing an additional 0.5 mg of pyridoxine; two participants did not receive B complex, and one received one tablet/day only.

The first 21 participants were cART-naïve due to the more restrictive inclusion criteria implemented during the pilot period (see Section 3.2.5); these participants have not been included in descriptions and analysis of baseline cART status. In subsequent participants, at baseline, cART was ongoing in 24 (25.3%) for a median duration of 20 days (Q1-Q3 4-120) (**Figure 4-3**). The majority of participants (90.5%) were receiving co-trimoxazole prophylaxis; those not receiving it did so presumably in omission as they were not receiving dapsone.

Routine laboratory test results for serum urea were available for 60 participants in whom the median level was 3.9 mmol/l (Q1-Q3 2.5-5.6); all were normal, aside for that of one participant who had HIV-associated nephropathy. Liver function tests other than ALT were available in a minority only. Hepatitis B surface antigen test results were available for 53 participants, and were positive in four (8.0%). HIV viral load assessments were not done or available for any participants.

Females differed from males in terms of baseline characteristics: females were younger (34.4 vs. 37.8; p=0.029), lighter (46.9 vs. 51.0 kg; p=0.005) and shorter (159 vs. 170 cm; p<0.001) (but BMIs were no different). They also had lower $CD4^+$ T cell counts (76 vs. 155 cells/μl^3; p=0.016) and lower haemoglobin levels (8.7 vs. 9.6 g/dl; p=0.040).

4.2.2 Baseline DSP

At baseline, DSP (≥1 neuropathic symptom plus ≥1 neuropathic sign) was present in 65 participants (56.0%) (**Table 4-2**). Using the more stringent criteria of ≥1 neuropathic symptom plus ≥2 neuropathic signs, the frequency of baseline DSP was 41 (35.3%). Based on the criteria presented in Section 3.2.3, baseline DSP was ascribed to HIV (HIV-DSP) in 31 (47.7%), to INH (INH-PN) in 24 (36.9%) and to ongoing ART (ATN) in three (4.6%); the aetiology was indeterminate in seven (10.8%). Of those classified as HIV-DSP, 17 (57.0%) reported neuropathic symptom onset concurrent with the onset of TB symptoms (but prior to the initiation of anti-TB therapy).

Neuropathic symptoms were severe by BPNS sensory grade (highest NRS score ≥7) in 53.9% of those participants with baseline DSP. The most commonly reported symptom was pain (69.2%), and the mean NRS score for this modality was 4.5 (SD 3.9). Examination findings suggested a primarily small fibre DSP (impaired pinprick sensation only) in 21.9%, a primarily large fibre DSP (impaired vibration sense and/or reduced or absent ankle reflexes) in 26.6% and mixed fibre involvement in 51.6%. Fourteen participants exhibited weakness of toe and ankle dorsiflexion – the weakness was mild (MRC grade 4) in 12 and moderate (MRC grade 3) in two. The mean TNS score was 6.1/20 (SD 3).

Six participants (5.2%) experienced isolated neuropathic symptoms (in the absence of neuropathic signs) of whom three experienced severe symptoms. Seven participants (6.0%) were asymptomatic at baseline but reported having had neuropathic symptoms previously (i.e. symptoms were no longer present

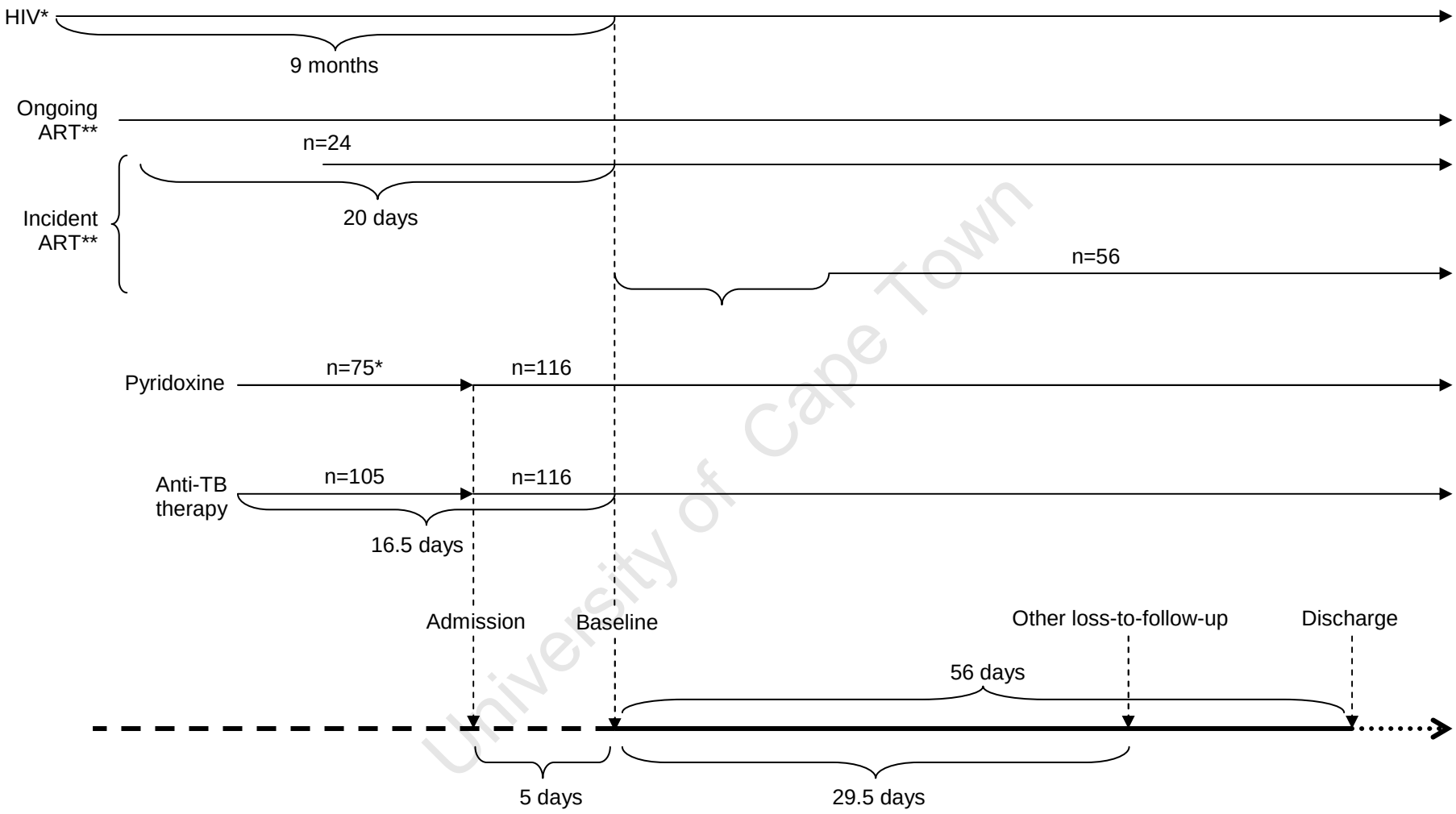


Figure 4-3 Timeline of HIV infection, anti-TB therapy, pyridoxine supplementation, antiretroviral therapy (ART) and hospital admission preceding (━ ━) and following (━━━) the baseline assessment. Note: all time values are medians. *Based partly on recall. **Relative to anti-TB therapy. (cf. **Figure 2-2**).

Table 4-2 Baseline neuropathic symptoms and signs.

Characteristic	Signs only	DSP	Symptoms only
n (%)	28 (24.1)	65 (56.0)	6 (5.2)
Symptoms			
Pain, n (%)		45 (69.2)	4 (66.7)
NRS, mean (SD)		4.5 (3.9)	2.7 (2.9)
Paraesthesia, n (%)		41 (36.9)	4 (66.7)
NRS, mean (SD)		3.9 (3.7)	3.5 (3.9)
Numbness, n (%)		41 (36.9)	5 (100)
NRS, mean (SD)		3.9 (3.5)	5.2 (3.1)
BPNS grade ≥3*, n (%)		35 (53.8)	3 (50.0)
Cramps**,***, n (%)		36 (55.4)	4 (66.7)
Signs, n (%)			
Impaired vibration	10 (35.7)	31 (47.7)	
Reduced/absent ankle reflexes	19 (67.9)	42 (64.6)	
Impaired pinprick	10 (35.7)	48 (73.9)	
Toe/ankle dorsiflexion weakness***	1 (3.6)	14 (21.5)	
Impaired proprioception**,***	6 (21.4)	30 (46.2)	
Small fibre signs only	6 (21.4)	14 (21.5)	
Mixed fibre signs	4 (14.3)	34 (52.3)	
Large fibre signs only	18 (64.3)	17 (26.2)	
TNS, mean (SD)	2.7 (1.7)	6.1 (3.0)	1.2 (0.4)

DSP=distal sensory polyneuropathy, NRS=numerical rating scale, SD=standard deviation, BPNS=Brief Peripheral Neuropathy Screen, TNS=Total Neuropathy Score.
*Equivalent to severe symptoms (highest NRS score ≥7).
**Not included in BPNS or TNS.
***Does not contribute toward DSP case definition.

at the time of the baseline assessment). Twenty-eight participants (24.1%) had neuropathic signs only (ADSP). Seventeen participants (14.6%) were entirely free of both neuropathic symptoms and signs.

Just over a third of those with baseline DSP (36.9%) had a diagnosis of neuropathy documented in the clinical notes – most were prescribed amitriptyline and half received higher dose pyridoxine (≥50 mg/day), as per the hospital protocol. Of the 49 participants with neuropathic pain (irrespective of DSP status), 19 (38.8%) were prescribed amitriptyline and one patient received tramadol. There was no significant difference in the overall subjective pain scores between those who were prescribed analgesia and those who were not (mean pain NRS 6.8 vs. 6.0; p=0.317).

4.2.3 Comparison of baseline characteristics

Participants with DSP at baseline were heavier than those without DSP, and a greater proportion had a BMI>18.5, but median heights were no different. Additionally, a greater proportion of those with DSP had extrapulmonary TB and a history of prior cART use, irrespective of current cART status. A greater

proportion of those with baseline DSP were receiving higher dose pyridoxine as per DPM protocol for DSP management (**Table 4-3**). Duration of anti-TB therapy was no different between the two groups.

Table 4-3 Comparison of characteristics of participants with and without DSP at baseline.

Characteristic	n=116	No DSP (n=51)	DSP (n=65)	*p*-value
Age, years, mean (SD)		36.5 (9.6)	35.4 (8.3)	0.589
Female, n (%)		27 (52.9)	37 (56.9)	0.669
Black, n (%)		22 (43.1)	39 (60.0)	0.123
Weight, kg, mean (SD)		46.1 (8.0)	50.8 (10.8)	0.013
Height, cm, median (Q1-Q3)	n=112	163 (156-168)	165 (157-170)	0.197
BMI, kg/m^2, mean (SD)	n=112	17.8 (3.9)	18.7 (3.4)	0.110
BMI >18.5, n (%)	n=112	36 (73.5)	35 (55.6)	0.051
Problem alcohol use, n (%)		20 (39.2)	21 (32.3)	0.434
Extrapulmonary TB, n (%)		13 (25.5)	28 (43.1)	0.049
Previous TB, n (%)		26 (51.0)	45 (69.2)	0.045
INH, mg/kg, median (Q1-Q3)		5.1 (4.6-5.5)	5 (4.7-5.4)	0.691
Pyridoxine ≥50 mg/day, n (%)		5 (9.8)	23 (35.4)	0.001
Prior pyridoxine, n (%)		33 (64.7)	42 (64.6)	0.992
WHO stage IV, n (%)		23 (45.1)	38 (58.5)	0.152
CD4$^+$, cells/µl^3, median (Q1-Q3)	n=114	110 (49-202)	84 (45-179)	0.306
CD4$^+$ <100, n (%)	n=114	21 (41.2)	35 (53.9)	0.175
Current cART use, n (%)	n=95*	10 (25.6)	14 (25)	0.944
Current d4T use, n (%)	n=95*	4 (10.3)	6 (10.7)	0.943
Previous cART use, n (%)		8 (15.7)	20 (30.8)	0.059
cART use ever, n (%)	n=95*	13 (33.3)	23 (41.1)	0.444
d4T use ever, n (%)	n=95*	6 (15.4)	11 (17.9)	0.751
Cr, µmol/l, median (Q1-Q3)	n=107	64 (55.5-72.5)	60 (49-81)	0.756
eGFR**, ml/min, median (Q1-Q3)	n=107	85.5 (63.3-106.9)	95.2 (72.0-127.9)	0.151
ALT, IU/l, mean (SD)	n=89	34 (47.5)	35.4 (5.8)	0.465
Hb, g/dl, mean (SD)	n=104	9.3 (2.0)	8.9 (2.1)	0.353
WCC, x 10^9/l, median (Q1-Q3)	n=103	5.4 (4.3-7.6)	5.7 (3.5-8.9)	0.860

DSP=distal sensory polyneuropathy, SD=standard deviation, Q1=1st quartile, Q3=3rd quartile, BMI=body mass index, INH=isoniazid, WHO=World Health Organisation, CD4$^+$= CD4$^+$ T cell count, cART=combination antiretroviral therapy, d4T=stavudine, Cr=creatinine, eGFR=estimated glomerular filtration rate, ALT=alanine transaminase, Hb=haemoglobin, WCC=white cell count.
*Pilot sample excluded.
**Calculated using the Cockcroft-Gault formula.

In those with DSP at baseline, DSP severity (as determined by TNS score, **Table 4-2**) correlated significantly with patient height (ρ=0.33; *p*=0.009). For the entire cohort, the TNS correlated with the period since diagnosis of HIV infection (ρ=0.24; *p*=0.027). There was an inverse correlation between numbness NRS score and number of years since the most recent TB episode (in those with a history of previous TB): participants with more recent infection had more severe numbness (ρ=-0.31; *p*=0.008).

Baseline characteristics differed between participants with INH-PN and those with HIV-DSP (**Table 4-4**). A greater proportion of those with INH-PN were

female, and had a history of previous TB. Participants with HIV-DSP were heavier and taller; a greater proportion had a $CD4^+$ T cell count <100 and a history of previous cART use. Participants with INH-PN experienced more severe pain than those with HIV-DSP (mean NRS 5.5 vs. 3.4; *p*=0.049).

Table 4-4 Baseline characteristics in participants with INH-PN vs. those with HIV-DSP*.

Characteristic	HIV-DSP (n=31)	INH-PN (n=24)	*p*-value
Female, n (%)	12 (38.7)	18 (75.0)	0.007
Weight, kg, mean (SD)	55.9 (9.9)	44.7 (9.3)	<0.001
Height, cm, median (Q1-Q3))	169.0 (163-177)	157.5 (156-169)	<0.001
BMI >18.5, n (%)	17 (54.8)	5 (22.7)	0.019
Previous TB, n (%)	19 (61.3)	21 (87.5)	0.030
$CD4^+$ <100, n (%)	21 (67.7)	8 (33.3)	0.011
Previous cART use, n (%)	13 (41.9)	4 (16.7)	0.044

HIV-DSP=HIV-distal sensory polyneuropathy, INH-PN=isoniazid-associated peripheral neuropathy, Q1=1st quartile, Q3=3rd quartile, BMI=body mass index, $CD4^+$= $CD4^+$ T cell count, cART=combination antiretroviral therapy.
*Significant differences only are presented.

Participants in the pilot study differed from the main sample in terms of ART use (by definition) and were younger (34.9 vs. 40.5; *p*=0.013).

4.2.4 Baseline risk factors

Univariate analysis revealed increasing weight and height, the presence of extra-pulmonary TB and a history of prior cART use to predict baseline DSP (**Table 4-5**). Cumulative neurological insults (1 point for each episode of TB, 1 point for any d-drug exposure and 1 point for a $CD4^+$ T cell count <100) was a significant predictor of DSP status (PR 1.1 per each additional insult; 95%CI 1.01-1.3). Multivariate analysis using generalised linear models was performed incorporating risk factors significant at a level of *p*<0.2 on univariate analysis (crude ratios were adjusted for age and gender). Each risk factor entered into the model is listed in **Table 4-5**. In the multivariate model, independent risk factors for DSP were female gender (PR 1.5; 95%CI 1.01-2.2), black race (PR 1.4; 95%CI 1.02-2.0) and increasing weight (PR 1.03 per 1 kg increase; 95%CI 1.0002-1.05).

Multivariate analyses were also performed using INH-PN and HIV-DSP as the dependent variables. Prevalence ratios with 95%CIs were calculated relative to DSP-free individuals (univariate data not shown). A history of previous TB was an independent risk factor for INH-PN at baseline (PR 4.2; 95%CI

1.20-14.8). Increasing weight was independently associated with HIV-DSP (PR 1.04; 95%CI 1.02-1.07).

Table 4-5 Univariate analysis of baseline DSP risk factors.

Covariate	n	% DSP	PR (95%CI)*
Age**			0.995 (0.98-1.01)
Female			
No	52	53.8	1
Yes	64	57.8	1.1 (0.8-1.6)
Black race			
No	54	48.1	1
Yes	61	63.9	1.4 (0.96-1.96)
Weight**			1.02 (1.007-1.06)
Height**			1.02 (1.003-1.05)
BMI>18.5, n (%)			
No	71	49.3	1
Yes	41	68.3	1.4 (0.99-1.9)
Previous TB			
No	45	44.4	1
Yes	71	63.4	1.4 (0.97-2.1)
Extrapulmonary TB			
No	75	49.3	1
Yes	41	68.3	1.4 (1.001-1.9)
WHO stage IV			
No	55	49.1	1
Yes	64	59.4	1.3 (0.9-1.8)
$CD4^+C<100$			
No	56	62.5	1
Yes	60	50.0	1.3 (0.93-1.8)
Previous cART			
No	88	51.1	1
Yes	28	71.4	1.4 (1.02-1.96)

DSP=distal sensory polyneuropathy, PR=prevalence ratio, BMI=body mass index, WHO=World Health Organisation, $CD4^+C$=$CD4^+$ T cell count, cART=combination antiretroviral therapy.
*Adjusted for age and gender.
**Per 1 unit increment.

4.3 Longitudinal analysis

4.3.1 Longitudinal characteristics

Participants that were lost to follow-up before the eight week assessment (see **Figure 4-2**) were heavier at baseline than those who remained in the study till at least eight weeks (50.6 vs. 46.6 kg; p=0.038) and had a higher mean BMI (19 vs. 17.4 kg/m^2; p=0.018). A higher proportion had a history of renal dysfunction (87.5% vs. 12.5%, p=0.045), but estimated glomerular filtration rates were not lower in this group. There were no significant differences in baseline DSP status between participants that were lost to follow-up before

the eight week assessment and those who remained in the study till at least eight weeks.

Of the 92 participants who were not receiving ongoing cART at the time of admission, 56 (61.0%) commenced cART during the course of the study; the median time to commencement of cART was 14 days (Q1-Q3 8-18) post-admission (**Figure 4-3**). Twenty-three (41.1%) commenced a d4T-based regimen, 19 (33.9) a TDF-based regimen and 14 (25.0%) an AZT-based regimen. Those who did not commence cART were either discharged before ART could be initiated, or did not satisfy national criteria for cART initiation. Twelve participants switched cART regimen during the course of the study, mostly (n=7) from a d4T-based to a TDF-based regimen. Switches were protocol-driven and not due to adverse events.

Patients switched to the continuation phase of anti-TB therapy (see Section 3.2.1.2) during the course of the study, but the INH dose remained unchanged. However, the effective INH dose, a function of body weight, increased as body weight and BMI increased through the course of the study, trend estimation revealing the increases to be statistically significant – for INH dose/body weight: $p<0.001$, for body weight: $p=0.027$ and for BMI: $p=0.047$ (for a description of trend estimation see Section 3.6.2.2).

Additional laboratory results were available for a minority of participants at follow-up as routine blood testing at DPM is usually done on admission only, unless clinically indicated.

4.3.2 Longitudinal DSP

4.3.2.1 Incident and worsening DSP

There were nine cases of incident DSP among the 37 participants who were DSP-free at baseline and had follow-up assessments. Based on the criteria presented in Section 3.2.3, incident DSP could be categorised as ATN in seven (77.8%) and INH-PN in two (23.2%). Four incident ATN cases were

receiving TDF, two d4T and one AZT. The incidence of DSP was 12.3/100 person-months.

Worsening DSP occurred in 21 of the 65 participants with baseline DSP; in 14 (66.7%), worsening occurred after initiation of cART. Six had been initiated on AZT, five on d4T and three on TDF. Worsening of baseline DSP occurred at a rate of 39.0/100 person-months. Incident DSP cases were evident throughout the study period (**Figure 4-4A**), while worsening DSP tended to occur soon after baseline (**Figure 4-4B**).

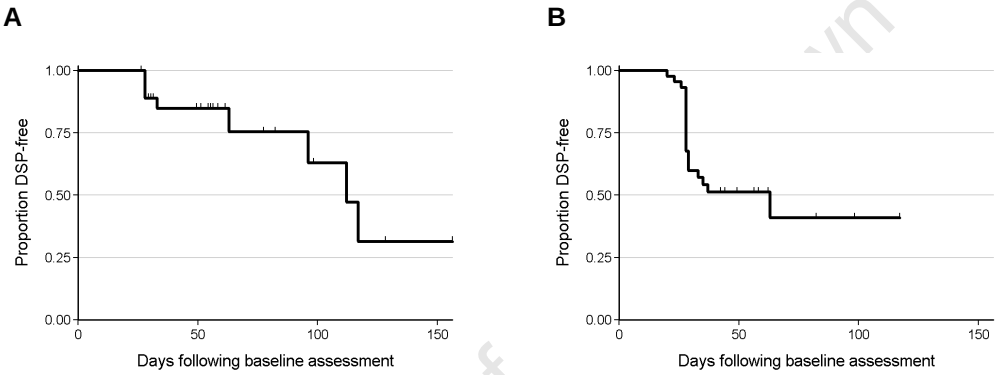


Figure 4-4 Kaplan-Meier survival plots for (**A**) incident and (**B**) worsening DSP with the origin of analysis at the baseline assessment. Wedges indicate censoring events.

4.3.2.2 DSP natural history

In participants with baseline DSP, neuropathic symptoms improved over 12 weeks (mean NRS scores for each modality decreased significantly), but the mean TNS score did not change significantly. For participants who were DSP-free at baseline, the mean TNS increased significantly, driven by increases in mean vibration and reflex scores (**Figure 4-5**).

Subjective changes in DSP severity were reflected in the PGIC scores for each time point (participants were asked to compare their current symptoms to symptoms present at the previous [as opposed to the baseline] visit). The scores were coded -2 for “much worse”, -1 for “worse”, 0 for “no change”, 1 for “better” and 2 for “much better”. The mean PGIC for each time point was consistently >0, thus indicating overall consistent improvement.

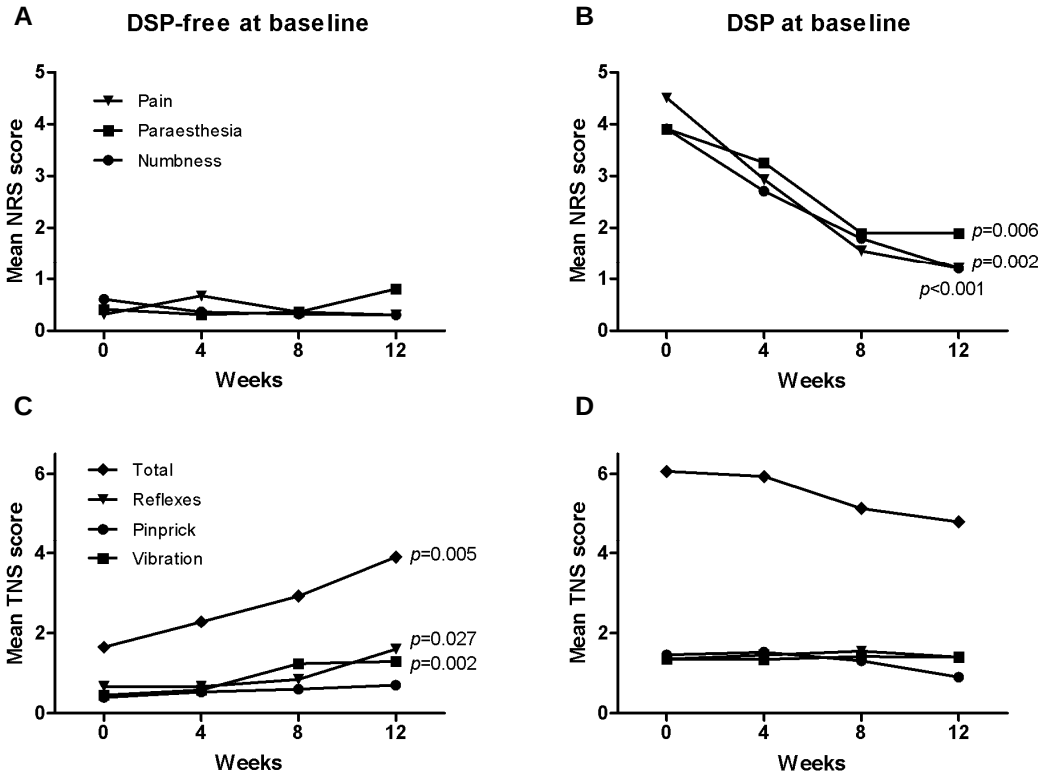


Figure 4-5 Symptom natural history over 12 weeks as demonstrated by change in the numerical rating scale (NRS) score for each modality of pain, paraesthesia and numbness in (**A**) participants DSP-free at baseline and (**B**) those with DSP at baseline. Total Neuropathy Score (TNS) and physical finding scores for each examination modality of reflexes, pinprick and vibration over 12 weeks in (**C**) participants DSP-free at baseline and (**D**) those with DSP at baseline. Note: n=116 at 0, n=80 at 4, n=54 at 8 and n=20 at 12 weeks. Error bars omitted for clarity. Trend analysis tested the significance of score changes over time.

4.3.3 Longitudinal risk factors

For the calculation of hazard ratios for incident DSP, the origin of the analysis was set at the date of anti-TB therapy initiation, which was treated as a virtual time point (a "left-shifted" survival analysis, see Section 3.6.2.2). Covariates were not time-varying – baseline values were used. A univariate analysis showed a clinical history of renal dysfunction, a history of prior TB, the presence of extrapulmonary TB, WHO stage IV HIV disease and the absence of cART at the time of anti-TB therapy initiation to be associated with incident DSP (**Table 4-6**). Risk factors significant at a level of $p<0.2$ (those listed in **Table 4-6**) informed a multivariate analysis: renal dysfunction (HR 4.2; 95%CI 1.2-14.4), prior TB (HR 6.6; 95%CI 2.5-17.4) and extrapulmonary TB (HR 2.7; 95%CI 1.01-7.2) were independently associated with incident DSP.

Table 4-6 Univariate analysis of incident DSP risk factors.

Covariate	p-m at risk	Events	Event rate	HR (95% CI)*
Age**				0.98 (0.95-1.02)
Female				
No	78.6	15	19.1	1
Yes	77.1	25	32.4	1.7 (0.9-3.4)
Renal dysfunction***				
No	150.9	36	23.9	
Yes	4.9	4	81.8	3.4 (1.1-10.4)
Previous TB				
No	68.1	11	16.1	1
Yes	87.6	29	33.1	1.8 (0.9-3.7)
Extrapulmonary TB				
No	34.4	24	19.8	1
Yes	121.4	16	46.5	2.6 (1.3-5.0)
WHO stage IV				
No	86.9	16	18.4	1
Yes	68.9	24	34.8	1.8 (0.95-3.4)
cART at anti-TB therapy start				
Yes	82.5	14	17.0	1
No	73.3	26	35.5	2.4 (1.2-4.8)
Haemoglobin**				0.8 (0.7-1)

p-m=person-months, HR=hazard ratio, WHO=World Health Organisation, cART=combination antiretroviral therapy.
*Adjusted for age and gender.
**Per 1 unit increase.
***Based on a history of renal dysfunction in the clinical notes.

Worsening of DSP could not be extrapolated to the virtual time point (as worsening was observed during the study period only), thereby precluding worsening DSP as an outcome in this analysis. When using the baseline assessment as the origin, there were no significant predictors for DSP worsening.

4.4 Vitamin B6 analysis

Plasma PLP and 4PA levels were determined in a pilot sample of 25 subjects at baseline, and again at one follow-up time point: 14 subjects had levels determined at the four week follow-up (group "A"), and the remaining 11 subjects at the eight week follow-up (group "B"). Neither baseline DSP frequency nor baseline PLP or 4PA levels were significantly different between the two groups (p=0.166, 0.434 and 0.373 respectively).

At baseline, the mean plasma PLP level was 95.7 nmol/l (SD 35.6). Follow-up PLP levels were higher than baseline levels; this difference was significant in group “B” (124.3 vs. 89.6 nmol/l; *p*=0.028) (**Figure 4-6**). Median 4PA levels did not differ between time points. PLP levels at all time-points were within the normal range (≥30 nmol/l) aside from those for one participant whose baseline PLP was 25.8 nmol/l (and 4PA 48.0 nmol/l). This subject had not received supplementary pyridoxine at the referring facility and was assessed on the day of admission. After eight weeks of pyridoxine 25 mg/day, the PLP level rose to 130 nmol/l, and 4PA to 430 nmol/l.

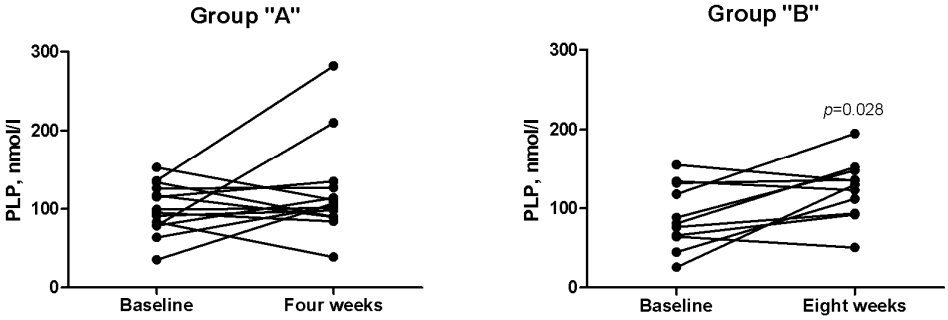


Figure 4-6 Pyridoxal 5‘-phosphate (PLP) levels at baseline and follow-up in groups “A” and “B”.

Plasma samples for vitamin B6 analysis were obtained a minimum of approximately five hours and a median of six hours (Q1-Q3 5.5-6.5) post-pyridoxine dosing; there was no correlation between number of hours post-dosing and PLP or 4PA levels. Participants receiving higher dose pyridoxine supplementation (≥50 mg/day) achieved higher baseline PLP levels than those receiving standard doses (25 mg/day) at baseline (116.4 vs. 87.6 nmol/l) – the difference was significant by single-tailed t-test only (*p*=0.034) (**Figure 4-7A**); however, a significant correlation between baseline PLP levels and cumulative pyridoxine dose was evident (ρ=0.480; *p*=0.014) (**Figure 4-8A**). The relationship between pyridoxine dose (current or cumulative) and PLP levels was not evident at follow-up; although follow-up PLP levels were inversely correlated with daily INH dose (ρ=-0.61; *p*=0.001) (**Figure 4-8D**). The relationship fell away after adjusting for weight, however. Median 4PA levels were significantly higher in those receiving higher dose pyridoxine at follow-up (1370 vs. 569.5 nmol/l; *p*=0.033) (**Figure 4-7D**).

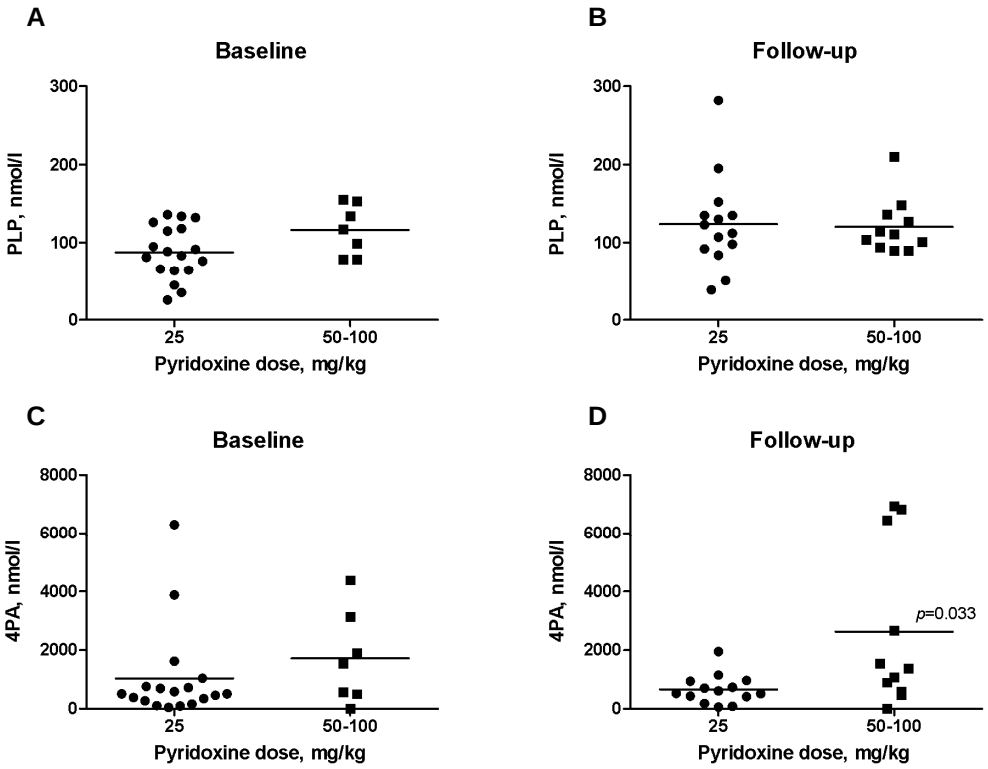


Figure 4-7 Pyridoxal 5'-phosphate (PLP) levels at (**A**) baseline, and (**B**) follow-up; and 4-pyridoxic acid (4PA) levels at (**C**) baseline and, (**D**) follow-up in those receiving standard (25 mg/day) and higher dose (≥50 mg/day) pyridoxine. Horizontal bar indicates mean for PLP and median for 4PA.

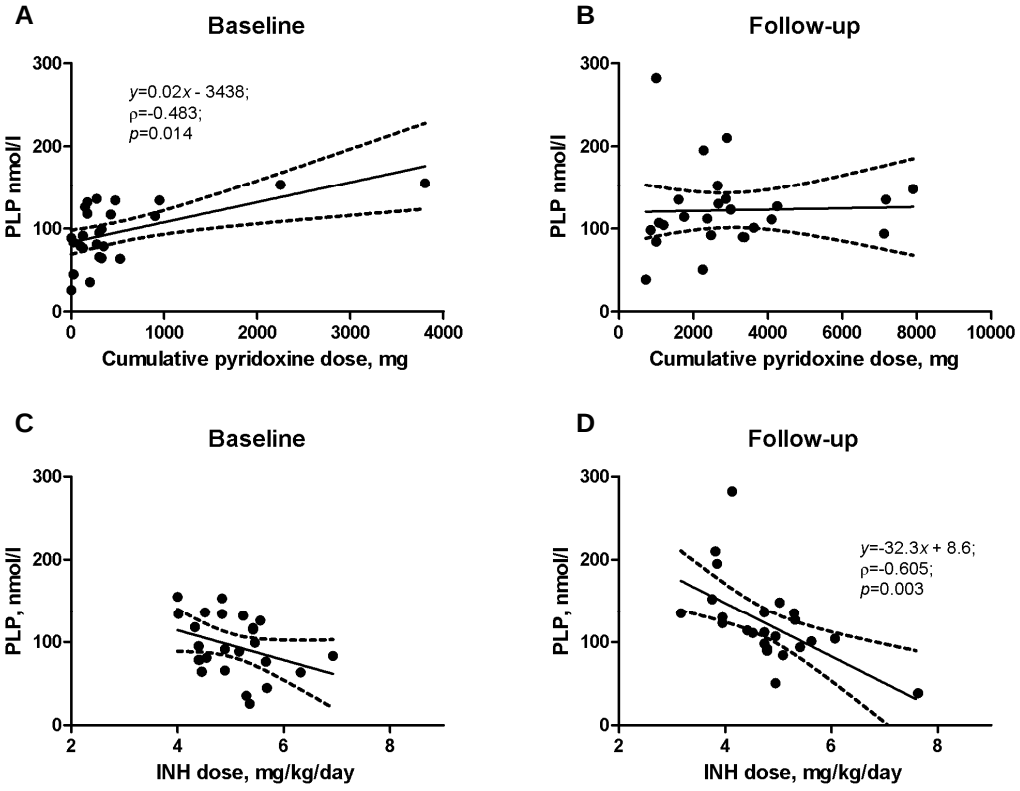


Figure 4-8 Pyridoxal 5'-phosphate (PLP) levels as a function of cumulative pyridoxine dose at (**A**) baseline, and (**B**) follow-up. PLP levels as a function of daily INH dose per body weight at (**C**) baseline, and (**D**) follow-up. Solid line indicates regression line, dashed lines indicate 95% confidence intervals

4.5 NAT2 genotyping and sequencing

4.5.1 Genotyping

Eighty-one participants were selected for genotyping on the basis of availability of follow-up data. Buffy coat was unavailable for one participant, and *NAT2* genotyping was performed on the remaining 80 participants.

4.5.1.1 PCR RFLP analysis

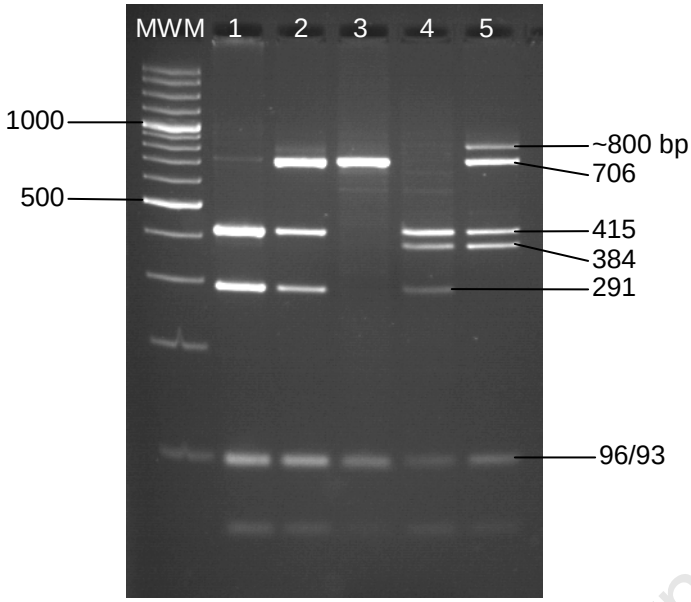
Extraction of DNA from buffy coat yielded genomic DNA in concentrations ranging from 20.5 to 630.1 ng/µml. Amplification by PCR resulted in products of ~895 bp, which were of good integrity.

RFLP analysis revealed the presence of the $G^{191}A$, $C^{282}T$, $C^{481}T$, $G^{590}A$, $A^{803}G$ and $G^{857}A$ *NAT2* SNPs at frequencies and as inferred from the products of restriction enzyme digestions "A"-"D" presented in **Figure 4-9** to **Figure 4-12**. The $A^{434}C$ and $A^{845}C$ SNPs were not detected in any samples.

Digestion of two samples (T059 and T103) in digest "A" yielded unexpected fragments of ~800 bp (**Figure 4-9**, lane 5), even after repeat DNA extraction, PCR and restriction enzyme digestion. Troubleshooting steps were taken to resolve the fragments (see Section 5.1.12).

4.5.1.2 Allele-specific PCR

Allele-specific PCR designed to detect mutations at the 341 locus revealed homozygosity for the wild type allele (341T) in 29 participants, while the remainder appeared heterozygous for the $T^{341}C$ SNP (**Figure 4-14A**). The lack of homozygosity for the mutation, which was out of keeping with Hardy-Weinberg equilibrium, suggested a lack of specificity in amplification of the 341 allele where the primer specific for the allele was employed (i.e. amplification occurred where the 341T allele was not present). After multiple unsuccessful attempts at optimising the reaction across temperature gradients and magnesium concentrations, we abandoned it in favour of sequencing.



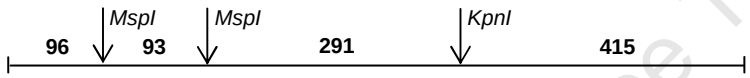
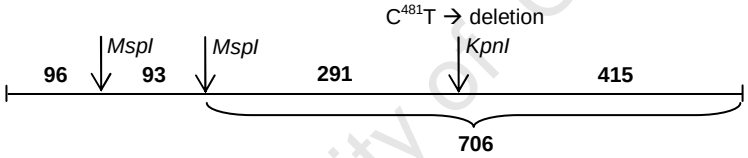
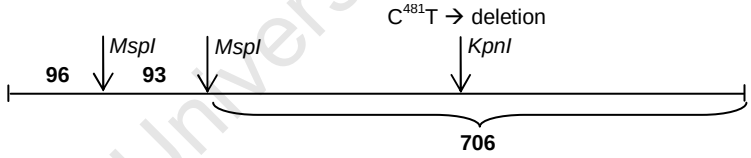
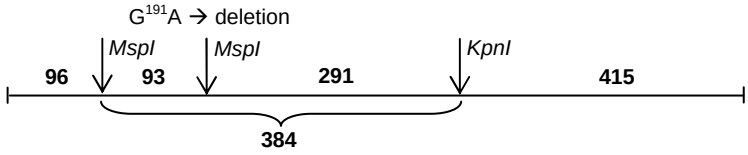
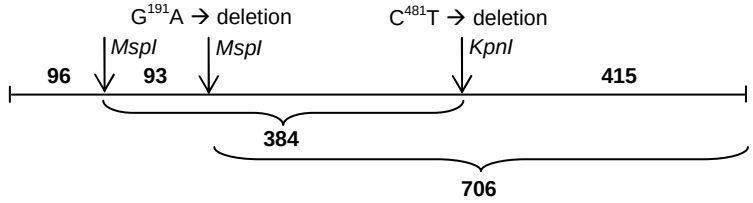
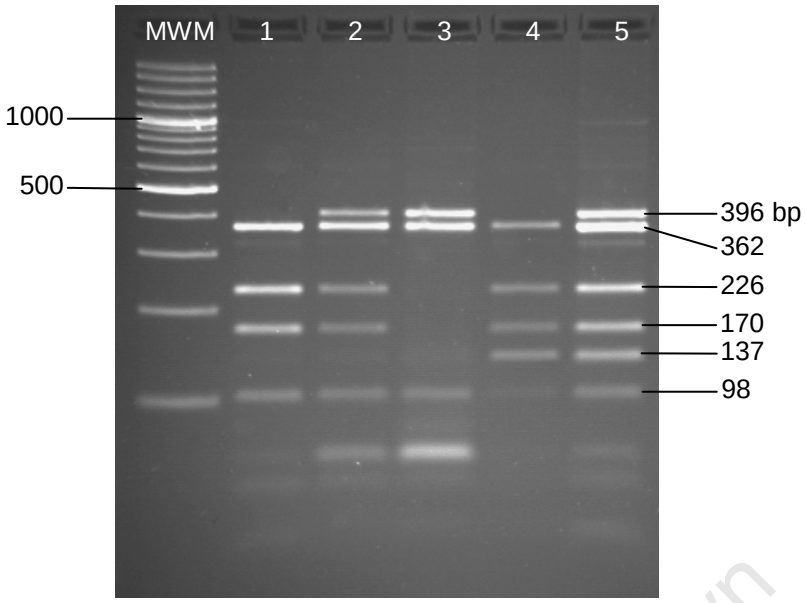
Lane	Schema of restriction enzyme digestion	SNPs	n=80 (%)
1		-/-	32 (40.0)
2		481/-	40 (50.0)
3		481/481	4 (5.0)
4		191/-	2 (2.5)
5		481/191	2 (2.5)

Figure 4-9 Single nucleotide polymorphism (SNP) combinations demonstrated in restriction fragment length polymorphism digest "A" (*MspI* and *KpnI*) along with illustrative digestion schema and frequencies of each combination. Note: the $A^{434}C$ SNP, which was not demonstrated in any sample, has been omitted for clarity. The bolded numbers are base pairs corresponding to the bands in the relevant lanes. MWM=molecular weight marker.



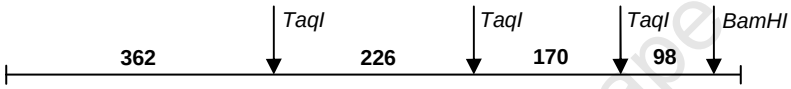
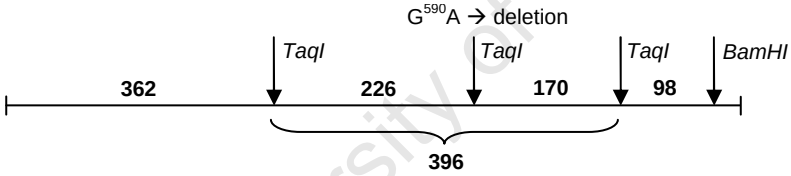
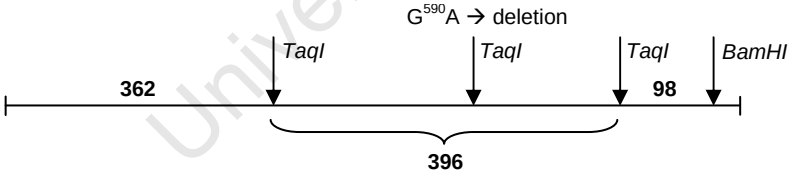
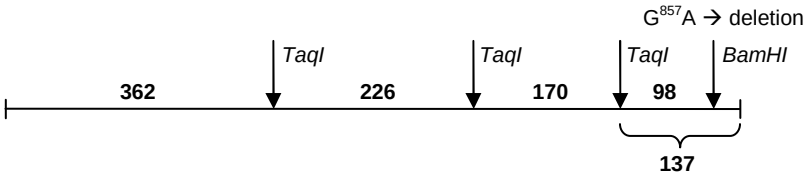
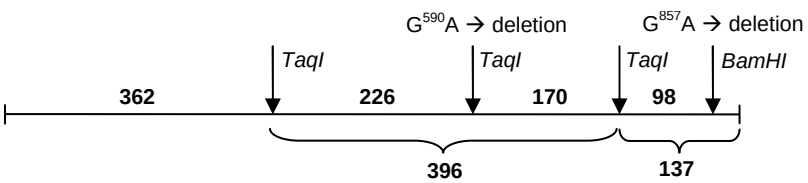
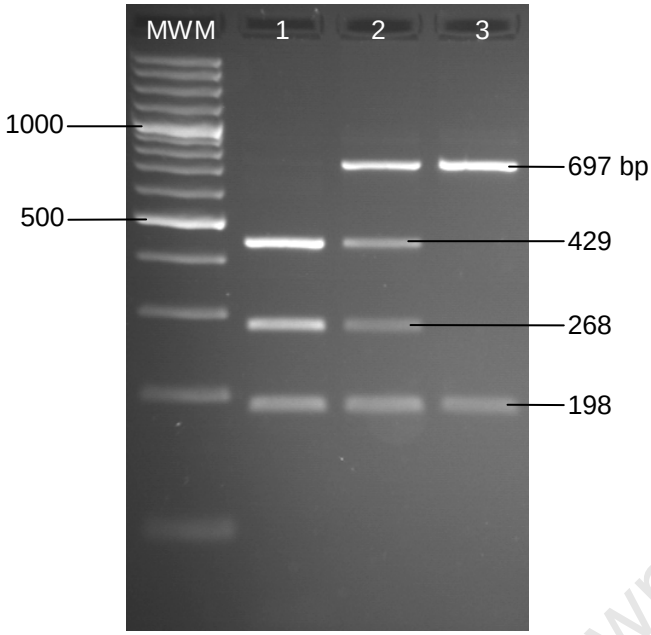
Lane	Schema of restriction enzyme digestion	SNPs	n=80 (%)
1		-/-	50 (62.5)
2		590/-	24 (30.0)
3		590/590	3 (3.75)
4		857/-	1 (1.25)
5		590/857	2 (2.5)

Figure 4-10 Single nucleotide polymorphism (SNP) combinations demonstrated in restriction fragment length polymorphism digest "B" (*TaqI* and *BamHI*) along with illustrative digestion schema and frequencies of each combination. The bolded numbers are base pairs corresponding to the bands in the relevant lanes. MWM=molecular weight marker.

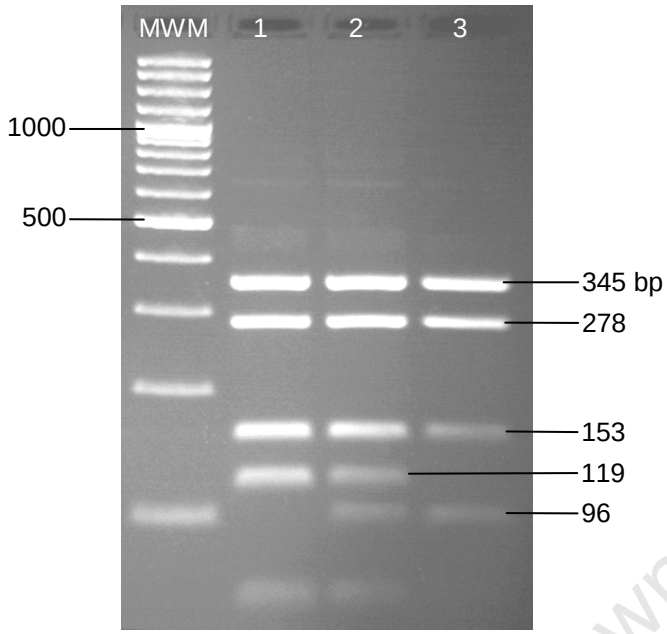


Lane	Schema of restriction enzyme digestion	SNPs	n=80 (%)
1	*FokI* (**268**) *FokI* (**429**) (**198**)	-/-	45 (56.25)
2	$C^{282}T$ → deletion; *FokI* (**268**) *FokI* (**429**) (**198**); **697**	282/-	24 (30.0)
3	$C^{282}T$ → deletion; *FokI* *FokI* (**198**); **697**	282/282	11 (13.75)

Figure 4-11 Single nucleotide polymorphism (SNP) combinations demonstrated in restriction fragment length polymorphism digest "C" (*FokI* and *DraIII*) along with illustrative digestion schema and frequencies of each combination. Note: the $A^{845}C$ SNP, which was not demonstrated in any sample, has been omitted for clarity. The bolded numbers are base pairs corresponding to the bands in the relevant lanes. MWM=molecular weight marker.

4.5.2 Sequencing

Sequencing in the forward direction was performed for the 51 samples appearing heterozygous for the $T^{341}C$ SNP on allele-specific PCR. It revealed homozygosity in seven, and heterozygosity in 44. Electropherograms displaying examples of each are presented in **Figure 4-14B** and **C**.



Lane	Schema of restriction enzyme digestion	SNPs	n=80 (%)
1	*DdeI* **153** *DdeI* **278** *DdeI* **345** **119**	-/-	12 (15.0)
2	$A^{803}G$ → addition; *DdeI* **153** *DdeI* **278** *DdeI* **345** *DdeI* **119**; **96**	803/-	41 (51.25)
3	$A^{803}G$ → addition; *DdeI* **153** *DdeI* **278** *DdeI* **345** *DdeI*; **96**	803/803	27 (33.75)

Figure 4-12 Single nucleotide polymorphism (SNP) combinations demonstrated in restriction fragment length polymorphism digest "D" (*DdeI*) along with illustrative digestion schema and frequencies of each combination. The bolded numbers are base pairs corresponding to the bands in the relevant lanes. MWM=molecular weight.

The two samples with ambiguous RFLP findings presented in Section 4.5.1.1 above were sequenced in both directions and confirmed heterozygous for each of the $G^{191}A$ and $C^{481}T$ SNPs (**Figure 4-13**). A further six random samples were also sequenced in both directions, in order to confirm RFLP findings: there was 100% concordance between sequencing and RFLP

findings for the $G^{191}A$, $C^{282}T$, $C^{481}T$, $G^{590}A$ and $A^{803}G$ SNPs; however, as the 857 locus was unclear in the majority of the electropherograms, the presence of the $G^{857}A$ SNP was confirmed in only one of three samples. No novel SNPs were identified, nor were instances of the $A^{434}C$ or $A^{845}C$ SNPs.

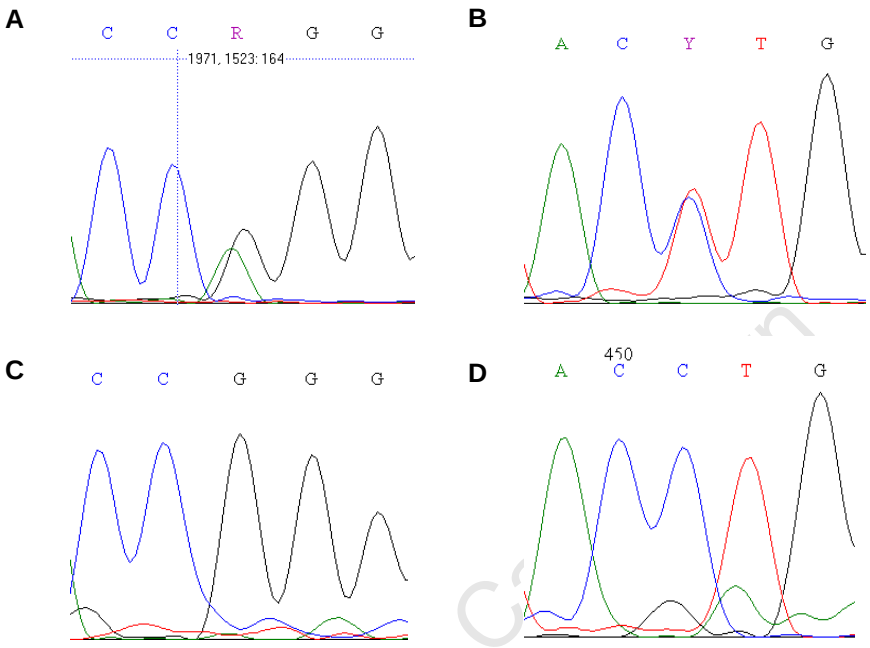


Figure 4-13 Electropherograms demonstrating heterozygous SNPs at the (**A**) 191 locus, and (**B**) 481 locus, for sample T059; and electropherograms demonstrating wild type alleles at the (**C**) 191 locus, and (**D**) 481 locus, for comparison.

4.6 Genetic data analysis and predicted phenotypes

4.6.1 SNPs

The most common SNP was the $A^{803}G$, while there were four instances only of the $G^{191}A$ and three of the $G^{857}A$. All SNPs other than the $C^{282}T$ and $C^{481}T$ were in Hardy-Weinberg equilibrium (p=0.017 and 0.047, respectively) (**Table 4-7)**. The lack of equilibrium for the $C^{282}T$ SNP was due to over representation of homozygous samples, five of which were sequenced and confirmed homozygous. For the $C^{481}T$, homozygosity was under represented, and again sequencing confirmed the genotype in 32 samples.

Individual SNP frequencies predicted neither baseline DSP nor INH-PN using generalised linear models to estimate prevalence ratios, nor incident DSP using proportional hazards models.

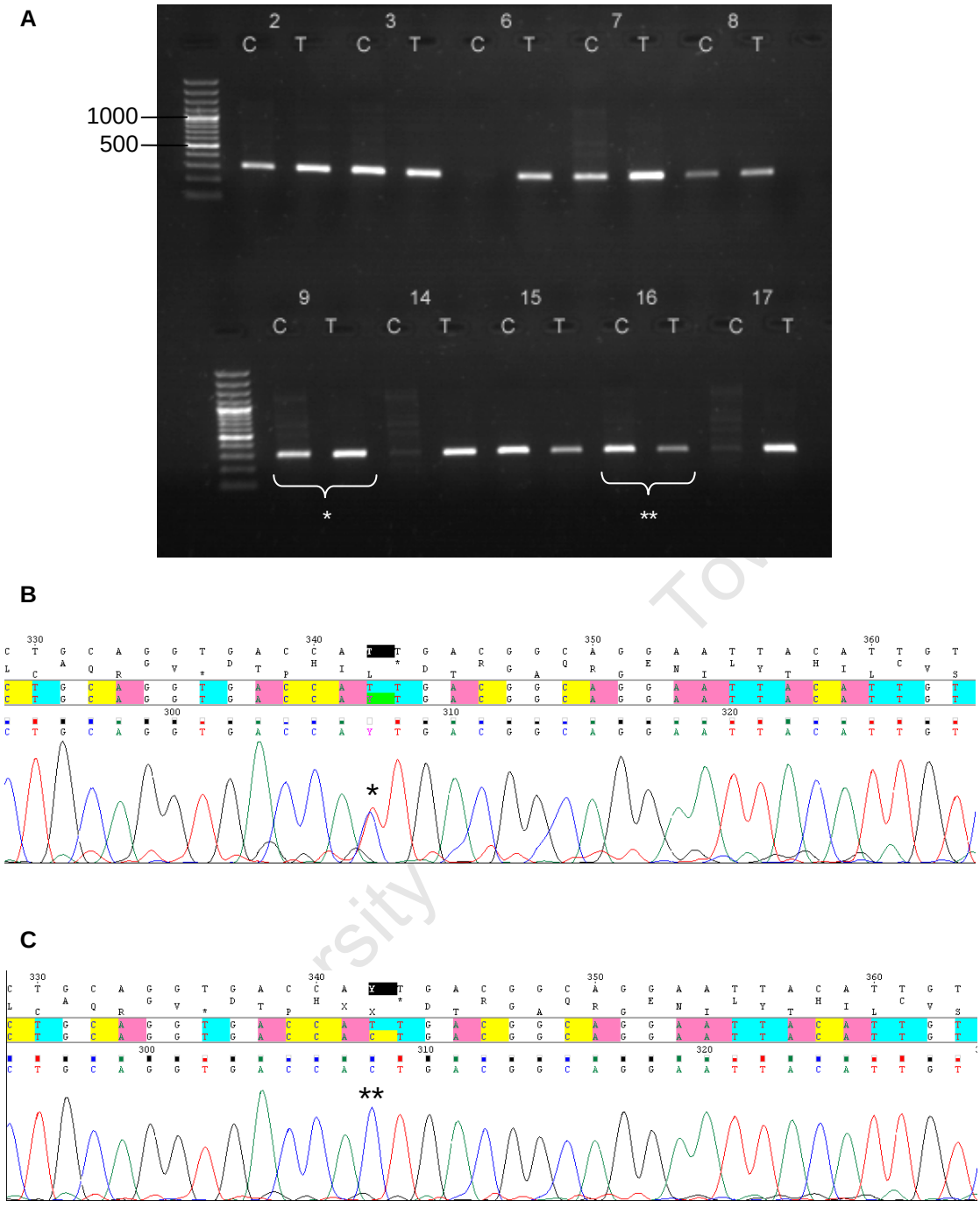


Figure 4-14 (**A**) Agarose gel of *NAT2* allele-specific PCR products demonstrating 341T homozygosity (in lanes 6, 14 and 17) and heterozygosity (in the remaining lanes) for the $T^{341}C$ SNP. (**B**) Electropherogram demonstrating heterozygosity for the $T^{341}C$ SNP, in agreement with allele-specific PCR (*). (**C**) Electropherogram demonstrating homozygosity for the $T^{341}C$ SNP, in contrast to the allele-specific PCR (**).

Table 4-7 SNP frequencies; frequencies of sample hetero- and homozygotes for each SNP; and associated Hardy-Weinberg equilibrium probabilities.

SNP	Frequency, n (%)	Heterozygotes, n (%)	Homozygotes, n (%)	*p*-value*
$G^{191}A$	4 (2.5)	4 (5.0)	0 (0.0)	0.819
$C^{282}T$	46 (28.8)	24 (30.0)	11 (13.8)	0.017
$T^{341}C$	58 (36.3)	44 (55.0)	7 (8.8)	0.089
$A^{434}C$	0 (0.0)	0 (0.0)	0 (0.0)	-
$C^{481}T$	50 (31.3)	42 (53.0)	4 (5.0)	0.047
$G^{590}A$	32 (20.0)	26 (32.5)	3 (3.8)	0.889
$A^{803}G$	95 (59.4)	41 (51.3)	27 (33.8)	0.577
$A^{845}C$	0 (0.0)	0 (0.0)	0 (0.0)	-
$G^{857}A$	3 (1.9)	3 (3.8)	0 (0.0)	0.864

SNP=single nucleotide polymorphism.
*As calculated using the Hardy-Weinberg equilibrium equation and the X^2 test with one degree of freedom.

4.6.2 Predicted haplotypes

Computational PHASE haplotyping revealed 14 *NAT2* haplotypes present in the study population (**Table 4-8**); the most frequent was *NAT2*5B* (28.1%), and wild type *NAT2*4* was present in 11.9%. The PHASE case-control function revealed no differences in haplotype frequencies between black and mixed ancestry participants (p=0.28), and between participants with and without baseline DSP (p=0.61). Attrition in the longitudinal data precluded use of the PHASE case-control function to detect differences in haplotype frequencies between those with and without incident DSP.

Table 4-8 Haplotype frequencies as predicted by PHASE computational haplotyping.

Haplotype*	*NAT2***	2n (%)***
000000000	**4*	19 (11.9)
001010000	**5A*	1 (0.6)
001010100	**5B*	45 (28.1)
001000100	**5C*	11 (6.9)
001001000	**5E*	1 (0.6)
010001000	**6A*	30 (18.8)
010011000	**6C*	1 (0.6)
010000001	**7B*	3 (1.9)
000000100	**12A*	33 (20.6)
010000100	**12B*	3 (1.9)
000010100	**12C*	3 (1.9)
010000000	**13A*	6 (3.8)
100000000	**14A*	1 (0.6)
110000000	**14B*	3 (1.9)

*Haplotype with nine positions: 191, 282, 341, 434, 481, 590, 803, 845 and 857.
**Standard nomenclature in Human NAT2 alleles (haplotypes)[139] (available from: http://n-acetyltransferasenomenclature.louisville.edu/).
***Two chromosomes per participant.

4.6.3 Predicted acetylation phenotypes

Figure 4-15A demonstrates the predicted NAT2 acetylation phenotypes for the 80 participants in whom genetic analysis was done. Nearly a quarter were ambiguous (either intermediate or slow) because of the presence of two or more loci heterozygous for a slow allele. Computational PHASE analysis revealed the phase of each of the 18 ambiguous genotypes. It uniformly placed slow alleles on opposite chromosomes, thereby predicting a slow phenotype in all 18 (**Figure 4-15B**).

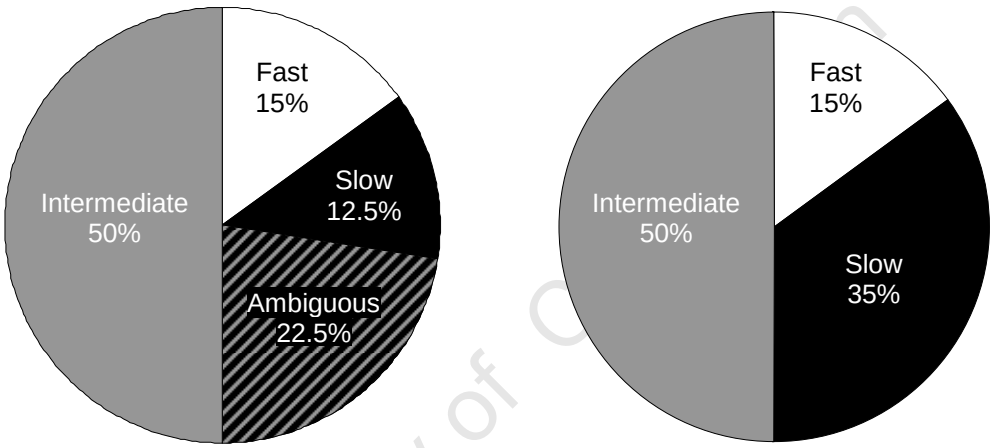


Figure 4-15 Overall predicted NAT2 acetylation phenotypes (**A**) before, and (**B**) after PHASE haplotyping. All ambiguous (either intermediate or slow) phenotypes were predicted to be slow.

Predicted phenotype frequencies did not differ between the black and mixed ancestry groups. At baseline, the proportions of slow acetylators in those with and without DSP did not differ (39.3 vs. 60.7%; *p*=0.451; PR 1.2; 95%CI 0.8-1.8); the proportions of slow acetylators in those with and without INH-PN also did not differ (57.9 vs. 42.1%; *p*=0.392; PR 1.4; 95%CI 0.7-2.9). DSP was not more severe in slow acetylators. There was a trend for slow acetylators to develop DSP on a survival analysis in which the origin was set to the date of anti-TB therapy initiation (**Figure 4-16**) (HR 1.6; 95%CI 0.8-3.4).

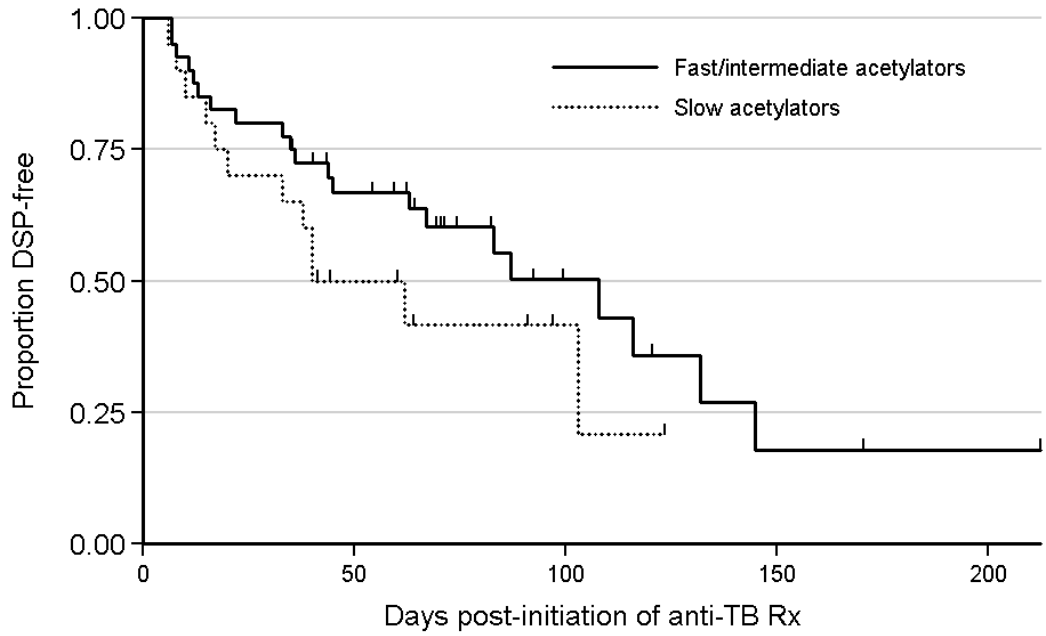


Figure 4-16 Kaplan-Meier plot of incident DSP in slow vs. fast/intermediate acetylators with origin at the date of anti-TB therapy initiation. Wedges indicate censoring events.

Both baseline and follow-up PLP levels failed to aggregate when grouped by slow vs. fast/intermediate acetylators (**Figure 4-17**); however, fast/intermediate acetylators achieved greater increases in PLP levels from baseline to follow-up compared to slow acetylators (97.1-128.1 vs. 90.1-99.3; *p*=0.043) (**Figure 4-18**).

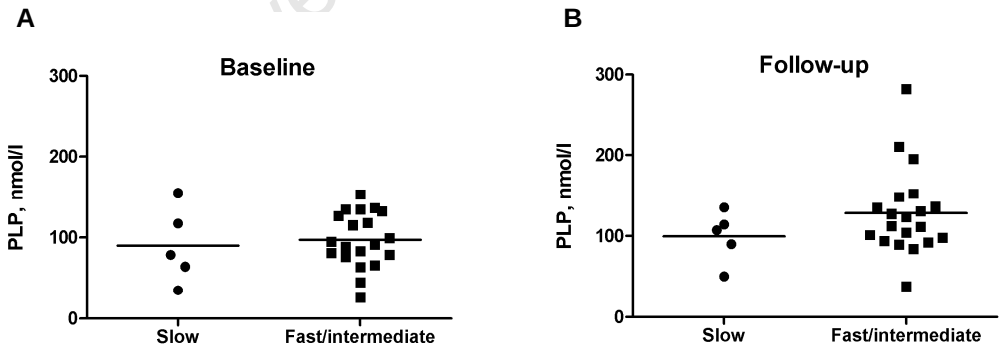


Figure 4-17 Pyridoxal 5'-phosphate (PLP) levels by predicted NAT2 phenotype (slow vs. fast/intermediate acetylation) at (**A**) baseline, and (**B**) follow-up. Solid line indicates mean.

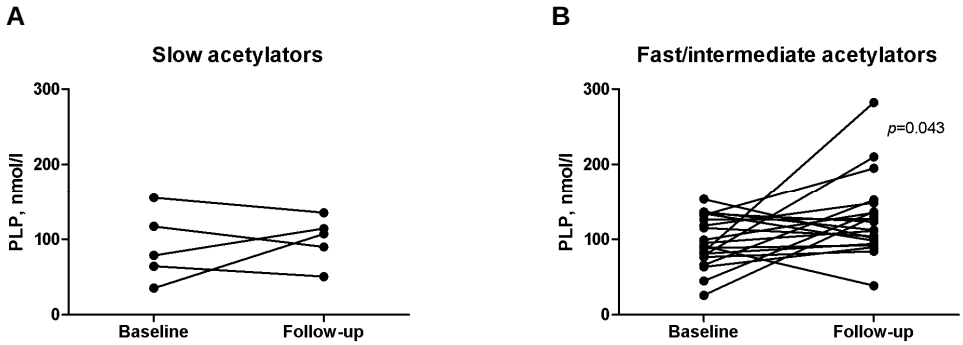


Figure 4-18 Pyridoxal 5'-phosphate (PLP) levels at baseline and follow-up in (**A**) slow acetylators and, (**B**) fast/intermediate acetylators.

5 Discussion

The discussion will explore and contextualise various aspects of the project before offering recommendations and presenting conclusions.

5.1 Observations

These are drawn into broad themes.

5.1.1 DSP is frequent after anti-TB therapy initiation and continues to develop during concomitant cART

A high rate of DSP was found in this inpatient co-infected cohort recently commenced on anti-TB therapy with INH. More than half (56%) demonstrated DSP at baseline; a further 24% demonstrated ADSP. The frequency of DSP is similar to the frequency of DSP in the HIV mono-infected d4T-treated outpatient cohort of Wadley et al. (57%)[12], and higher than the 37% demonstrated in a mixed HIV±ART, but geographically similar, outpatient population in Maritz et al.[11]. The entire cohort in Wadley et al. had been exposed to d4T; a high rate of DSP is therefore expected. The cohort of Maritz et al. was 50% ART-untreated, perhaps accounting for the lower prevalence. Observed rates of DSP are dependent on the criteria used to define a case of DSP[11]; differences in BPNS execution and interpretation may also have contributed to the discrepancy (see Sections 5.1.10). DSP rates may also differ between well-resourced and resource poor settings, hence the choice of comparison studies above. There are no prevalence data specific to HIV/TB-associated DSP with which to compare study findings. (Cumulative incidence data exist, but this analysis was precluded because of attrition.)

Incident DSP in this HIV/TB cohort was higher than that reported in Lanternier et al.[4] (12.3 vs. 5.4/100 person-months). The discrepancy may be attributed to prospective versus retrospective study, or use of the BPNS/TNS versus the criteria utilised in Lanternier et al. (see **Table 2-1**). Worsening DSP occurred at a higher rate, and followed a pattern distinct to that of incident DSP – worsening occurred early during the study period, while incident DSP

occurred throughout the study period. Worsening DSP may not be equivalent to incident DSP, a concept explored further in Section 5.1.11.

Participants with DSP experienced significant symptomatology; a large proportion (54%) experienced severe symptoms by BPNS criteria (highest NRS score ≥7) at baseline. The mean TNS score, which is indicative of DSP severity, was similar to that quoted in Robinson-Papp et al.[63] (3.9 vs. 3,7) when a similar patient group and definition of severity was utilised. (For direct comparison, the data were adapted to match Robinson-Papp et al. in terms of a combined ADSP/SDSP group, and a 16-point score in which neuropathic symptoms were excluded.) Patients with co-morbidities, such as those represented in both of these studies, may experience more severe neuropathic pain[167].

5.1.2 HIV-DSP, ATN and INH-PN, and possibly TB, all contribute to HIV/TB-associated DSP

Aetiological diagnosis was based on recall and temporal association (see **Figure 2-2**), an approach supported in the literature[54], but subject to recall bias. However, my impression was that participants readily recalled the onset of their symptoms relative to the initiation of anti-TB therapy or cART, and they were, on the whole, certain of their recollections. Furthermore, the intervening period was usually short, on the order of weeks. Another reassuring point was that HIV-DSP, as categorised using this approach, associated with some factors congruent with the literature, such as lower $CD4^+$ T cell count and height (see Section 5.1.3).

At baseline, the majority of participants had their DSP attributed to either HIV or INH; the contribution of ART was minimal – ATN was present in 5%. Only 20% of the whole cohort were receiving cART at baseline. A noteworthy finding was that INH-PN was more painful than HIV-DSP. A similar finding in Maritz et al. showed that ATN was more severe than HIV-DSP[11] – comparable rates of DSP (see above) and a similar severity profile may suggest that INH-PN and ATN are pathogenetically similar, and may both represent uncovering of subclinical HIV-DSP, driven by oxidative stress. Their

pathological features (see Section 2.2.3.2) may also support this hypothesis. However, more recent symptom onset in those with INH-PN may also explain the greater severity – our study demonstrated that neuropathic pain showed a steady decrease in severity over the period of observation.

The presence of a possible "TB-DSP" is a novel finding inferred from a history of neuropathic symptom onset concurrent with TB symptom onset. A literature search produced just three references to this phenomenon pre-HIV, the most recent in 1959[76] and the earliest in 1913[185], none of which reported any case studies or systematic study of the phenomenon. The paucity of literature can be attributed to either the non-existence of a "TB-DSP" entity, or INH-PN obscuring its incidence. The "TB-DSP" observed in this study may be limited to those co-infected with HIV – two contemporary observations are within this context[5,32]. Again, it may be that systemic inflammation accompanying TB infection drives oxidative stress and exacerbates HIV-related nerve injury. An implication is that not all cases of DSP occurring during anti-TB therapy are attributable to INH – the factors driving "TB-DSP" may still be present during therapy.

The aetiological diagnosis was ambiguous when the onset of DSP occurred during concomitant cART and anti-TB therapy. During follow-up, ATN was defined when incident or worsening DSP occurred at any point after cART initiation (see **Table 3-4**), but ART may not necessarily have been implicated. Nevertheless, most cases of incident or worsening DSP (70%) occurred after cART-initiation, suggesting that ART is an additional driving force behind incident/worsening DSP. ATN was defined even when "non-neurotoxic" agents, such as AZT or TDF, were prescribed. Antiretroviral agents traditionally thought to be non-neurotoxic may have their neurotoxicity profile altered by the presence of TB and/or anti-TB therapy. In addition, DSP incidence during TDF therapy is low but not negligible[186]. DSP was associated with cART independently of d-drug use in one study[13].

5.1.3 Previous neurological insults predict HIV/TB-associated DSP

This study supports the view that HIV-associated DSP is a multi-factorial process and that neurological insults to the peripheral nervous system are

cumulative (see Section 2.3.5). Previous TB, implying INH exposure, has previously been identified as a risk factor for ATN[11] and was similarly found to be a predictor of INH-PN in this study (again pointing to a similar pathogenetic risk profile). Previous exposure to low-dose INH is known to have a priming effect whereby an increase in INH dosage results in earlier-onset neuropathy compared with that seen in previously unexposed individuals[23]. Conversely, symptomatology may improve gradually after exposure to INH – one study finding was that numbness severity was inversely correlated with the latent period since previous TB, a finding corroborated in this study. There was no correlation between DSP and dose or duration of current INH therapy, arguing against cumulative toxicity of the agent. Previous, but not current, cART use was associated with HIV-DSP; ongoing cART may be protective for DSP (see Section 2.1.3), while previous exposure may have been of short duration, with neurotoxicity outweighing neuroprotection. Also, previous cART likely encompassed a d4T-based regimen (national protocols changed during the study period, see Section 3.2.1.2).

A novel approach in this study was the use of an "insult score" as a proxy for cumulative nerve injury (see Section 4.2.4), which produced significant results: each additional insult (each TB episode, exposure to d-drugs or a $CD4^+$ T cell count <100) increased DSP probability by 10%. The "insult score" was not entered into the multivariate analysis, however, because it compressed and categorised important covariates. Renal failure is a known cause of neuropathy, possibly explaining the association with incident DSP. However, the eGFR and creatinine were normal; renal dysfunction may therefore have been a proxy for advanced illness.

Not all neurological insults could be accounted for. Alcohol abuse is a known cause of DSP, but has not yet been shown to increase the risk for HIV/TB-associated DSP (see Section 2.1.3). The lack of association may be due to the measure used to assess alcohol use and abuse. For example, an overly inclusive definition of alcohol use can potentially underestimate its impact as an additional risk factor for DSP[11]. More accurate measures are not convenient[179], and may not delineate the pattern of alcohol use that drives

peripheral nerve injury. Within the literature surrounding HIV-associated DSP, various definitions have been utilised: any alcohol consumption in the prior 12 months[11], >30 standard drinks in one month[35] and any alcohol-related complication in the previous six months[101]. We used a simple validated single question screen to identify problem drinking in the study population[179] (see Section 3.3.5). The instrument demonstrated problem drinking in a high proportion; however, there was no association between problem drinking and DSP risk; a lack of specificity may be implicated. The impact of nutritional factors other than B6 could not be ascertained.

Factors suggesting advanced HIV/TB disease were also contributory. The association of HIV-DSP with lower $CD4^+$ T cell counts is not surprising. WHO HIV stage IV disease was also predictive of incident DSP on univariate analysis, as was the presence of extrapulmonary TB. Patients with extrapulmonary TB may experience greater systemic inflammation and the resulting oxidative stress may contribute to DSP pathogenesis. An indicator of general clinical condition such as the Karnofsky score may have been a useful covariate, but no data specific to general condition were collected.

Although gender differences have been demonstrated in other studies[55,187], the observed higher proportion of females with INH-PN when compared to HIV-DSP should be treated with caution – gender bias was a prominent feature of this study (see Section 5.1.8). Genetic disposition may explain the independent association of black race with baseline DSP[63]; however, *NAT2* genetic variation is not implicated. Further genetic or genomic investigation of the sample may elucidate the relationship.

Increasing height was a risk factor for baseline DSP, a finding corroborating that of previous research in HIV populations[12,55], and consistent with pathogenic factors such as mitochondrial mutation load (see Sections 2.3.2.1.3 and 2.3.2.2.2); however, the increased risk was small (2% increase per cm) with a wide confidence interval, and fell away on multivariate analysis. An association with height was more evident when HIV-DSP was compared to INH-PN: participants with HIV-DSP were significantly taller than those with

INH-PN (**Table 4-4**). HIV-DSP may be more length-dependent than INH-PN, the onset being slow and insidious in the former, and rapid in the latter. Additionally, increasing height correlated with increasing DSP severity (as estimated by TNS score), a novel finding in this study.

The independent association of increased weight with both general DSP and HIV-DSP in this cohort was surprising. DSP is associated with the metabolic syndrome (see Section 2.3.4.3), but participants in our study were not obese. Height may plausibly have accounted for increased weight, but the association with height fell away on multivariate analysis. Participants with HIV-DSP were heavier and had higher BMIs than those with INH-PN; again this finding was unexpected – HIV-DSP is associated with markers of advanced HIV infection, of which wasting is one. That a greater proportion of those with HIV-DSP were male and had a history of ART use (which may have improved clinical condition and therefore weight), and a lesser proportion had a history of previous TB when compared to those with INH-PN, may explain the weight difference.

The prevalence ratio, as estimated by generalised linear models, rather than the more widely utilised odds ratio, as estimated by logistic regression, was used to identify predictors of baseline DSP. The appropriateness of the odds ratio to describe cross-sectional data is questioned – while it does tend to the prevalence rate ratio (the gold standard predictor of risk) for rare outcomes, it overestimates the prevalence rate ratio when the outcome is frequent – it is better suited to case-control studies. The prevalence ratio is a better approximation of the prevalence rate ratio for frequent outcomes in cross-sectional data. Furthermore, interpretation of the odds ratio is conceptually complex when compared to the more intuitive prevalence ratio[188].

5.1.4 The natural history of DSP in HIV/TB shows overall improvement over time

Two analyses pointed to progressive improvement in baseline DSP through the course of the study: trend analysis of symptom NRSs (**Figure 4-5B**) and a consistently positive mean PGIC score (see Section 4.3.2.2). Despite initial

worsening of baseline DSP (possible “coasting”, see Section 2.2.5.3), overall improvement occurred in nearly half of all baseline DSP participants with follow-up data. Whether improvement occurred because of pyridoxine supplementation, improvement in general condition/immunological status or attrition bias, or because it is simply reflective of DSP natural history, is not clear. Formal analysis was prevented by heterogeneity in the cohort, confounding factors and attrition, as well as the complex statistical methods required.

The natural history of neuropathic signs differed between participants with baseline DSP compared to those DSP-free (i.e. those not satisfying criteria for baseline DSP, but with primarily ADSP [see Section 5.1.9]): on average the scores remained constant during follow-up in those with baseline DSP, while scores for vibration sense and reflexes in the DSP-free group worsened significantly (**Figure 4-5C** and **D**). ADSP progression therefore appears to involve predominantly large fibres, suggesting a pathogenetic process different to that for symptomatic DSP, and reinforces that clinically relevant (i.e. painful) DSP is small-fibre-dependent. It also may further refute the hypothesis that HIV-associated DSP exists as a spectrum between symptomatic and asymptomatic neuropathy[33,79,97]. On the other hand, seven of nine cases of incident DSP in this study were technically progressions from ADSP to DSP, and of whom six demonstrated only large fibre signs at baseline. Interpretation of the trend analyses should also be treated cautiously – bias may have accounted for the findings: for example, the decrement in mean scores could be a result of retention of participants with less severe symptoms. Study of ADSP natural history requires a cleaner cohort, and a larger sample size.

Four case histories provide further insight into the natural history of HIV/TB-associated DSP, and are presented in Appendix 4.

5.1.5 Pyridoxine supplementation may prevent B6 deficiency expected in the study population, but prior deficiency may have been missed

All but one of the 25 participants who had B6 status assessments were receiving pyridoxine supplementation at the time of the baseline assessment – some for several weeks and others for several days – and all were receiving pyridoxine at the follow-up assessment (which was either at four weeks [group ”A”] or eight weeks [group “B”]). Active pyridoxine supplementation accounts for the observed elevated PLP and 4PA levels (see below), and, at doses ranging from 25-100 mg/day, was sufficient to prevent or correct the deficiency expected in this population. As we were not able to sample prior to the initiation of supplementation, and had no controls, this statement is inferred – existing literature clearly points to prevalent B6 deficiency in HIV/TB populations in the absence of supplementation (see Section 2.3.4.1.1). The one participant who had not received pyridoxine at the time of the baseline assessment had levels below the reference range utilised (≥20 nmol/l), but not if the less conservative ≥30 nmol/l cut-off was utilised[153]. The patient did not have DSP, and could not provide power for comparison.

One concern was that the observed elevated PLP levels could be explained by the pharmacokinetic peak of the supplement, rather than steady-state plasma concentrations. Krishnamurthy et al. showed that vitamin B6 status was artificially inflated when sampling was performed 1-4 hours after pyridoxine dosing[26]. In the current study, the period between dosing and sampling exceeded five hours at all time points, which, according to one pharmacokinetic study[162], is the point at which PLP levels approach baseline, and which may then exclude the possibility that we were measuring the pharmacokinetic peak rather than stable baseline levels. If we were truly measuring the pharmacokinetic peak we would also expect there to be some correlation between plasma levels and hours since dosing, approximating the plasma concentration curve as it returns to baseline; however, our findings did not show any correlation. Vitamin B6 metabolism and pharmacokinetics may be altered in the presence of disease states, although the literature is deficient

in this respect[163,173]. Pre-dosing levels on fasted patients, the ideal sampling schedule, was not a viable possibility in this study. However, the pattern of plasma B6 markers in this study was reflective of ongoing and recent supplementation: PLP levels were 5-fold that defined as the lower limit of normal, and 4PA levels were 4-fold the upper limit of normal and >150-fold the lower limit[154,189].

Higher dose pyridoxine did not translate into higher PLP levels at either baseline or follow-up, while at follow-up, higher dose pyridoxine resulted in higher 4PA levels. These findings are consistent with the literature: urinary 4PA correlates with administered pyridoxine dose, while PLP levels plateau at higher doses; excess unbound PLP is rapidly oxidised to 4PA[154,161,190]. However, at baseline, PLP levels did correlate with cumulative pyridoxine dose, a function of both dose and period of supplementation. By follow-up, this correlation fell away; it is possible that PLP levels as a function of supplementation had achieved steady-state at the time. PLP levels increased significantly between baseline and eight weeks (see below). PLP levels at follow-up were inversely correlated with INH dose per body weight, but the association fell away after adjusting for weight. INH per body weight is mainly a function of weight as the numerator (i.e. absolute dose) varies little (4-6 mg/kg).

That higher doses of pyridoxine are more protective for DSP was not a testable hypothesis in this study because pyridoxine doses were increased by clinical staff in response to the diagnosis of symptomatic DSP. Furthermore, if DSP was identified by the study team, this was noted in the clinical records. In this way the study outcome influenced the exposure of interest.

One caveat is that group “A” and group “B” were combined into a single “follow-up” group, but levels at these time points may not be equivalent. When comparing baseline to follow-up levels in each group, a significant increase was seen in group “B” but not in “A”. As the two groups were mutually exclusive, comparing four week levels to eight week levels was precluded (repeated measures implies that sampling occurs in the same subjects).

Lack of pyridoxine supplementation at the start of anti-TB therapy and resulting B6 deficiency may have contributed to DSP risk; however, a history of pyridoxine supplementation prior to admission predicted neither baseline nor incident DSP, nor PLP levels. Unfortunately, data regarding prior supplementation are not reliable because they were dependent on participant recall and/or the accuracy of the referral letter – recall bias may have obscured any association. A poor compliance history may also have contributed to unreliability.

Vitamin B-complex supplementation was virtually universal within the cohort and was therefore not expected to influence the analysis. The vitamin B6 constituent of B-complex is variable between brands; to my knowledge, the B-complex administered to patients at DPM is LEN-B Co® which contains pyridoxine 0.5 mg/tablet (other brands may contain 2 mg/tablet). The contribution of this small amount of pyridoxine is negligible in the context of pyridoxine doses ≥25 mg. Consequently, vitamin B-complex was disregarded as a covariate and a total pyridoxine dose not calculated. Other research within our group shows that vitamin B-complex alone is insufficient to prevent HIV-associated DSP[112].

Because of the stated problems with sampling, and the apparent influence of supplementation inflating plasma PLP levels, we did not send further samples for B6 HPLC. Recommendations based on this evidence should be cautious.

5.1.6 *NAT2* genetic variation and predicted phenotypes in this Western Cape study population were described

Individual SNP frequencies were similar to those reported elsewhere for Western Cape black and mixed ancestry populations (for the SNPs reported in Adams et al.: $G^{191}A$, $C^{481}T$, $G^{590}A$ and $G^{857}A$)[141]. Predicted phenotypes also matched closely those of Parkin et al.[119]. Haplotypes have not been described for the Western Cape predominantly Xhosa and mixed ancestry “coloured” populations (or for any other South African population). Because SNP and haplotype frequencies did not differ between the two groups, they were

considered and analysed together. The Western Cape mixed ancestry population is derived from multiple origins (see Section 2.3.2.1.1); however, the contribution of African ancestry to *NAT2* genetic diversity may be overrepresented.

As we expected, the frequency of the wild type *NAT2*4* haplotype was low. *NAT2*4* is arbitrarily assigned based on its frequency in white populations, and is not common in African populations[116]. PHASE predicted 14 different haplotypes present in the population, including some at very low frequencies. This genetic diversity is representative of that found in Africa as a whole. Haplotypes in this study were also similar to those of some other sub-Saharan African populations, most notably the Gabonese Bantu in terms of *NAT2*5B*, *NAT2*6A* and *NAT2*12B*[191]. Again this is not surprising as the Xhosa are a Bantu people.

Two of the nine *NAT2* SNPs typed in this study ($C^{282}T$ and $C^{481}T$) were not in Hardy-Weinberg equilibrium. Lack of equilibrium could be attributed to the fact that the sample was not a random representation of the population – selection occurred not only on admission and enrolment (see Section 5.1.8), but also because NAT2 acetylation status was determined in only a subset of the cohort (70%) (those with any longitudinal data). As there was 100% agreement between RFLP analysis and sequencing for these two SNPs (aside from two samples in which RFLP findings were ambiguous, rather than conflicting, see Section 5.1.12), this would not explain the lack of equilibrium. Similar reported frequencies of the $C^{481}T$ SNP in Adams et al. (the $C^{282}T$ SNP was not typed in this study) was reassuring[141]. The PHASE analysis assumes Hardy-Weinberg equilibrium; it is not possible to gauge the impact of non-conformity to this assumption on the derived haplotype frequencies in the absence of other haplotype data for the population. PHASE haplotyping of *NAT2* in other populations has previously been shown to be in agreement with true haplotype 99.2% of the time[146].

5.1.7 There was a trend for slow acetylators to develop DSP

A trend for the slow acetylation phenotype to predict decreased DSP-free survival was evident on a survival curve, but was not significant either by the log-rank test for equality of survivor functions or by proportional hazards models. A reduced sample size (70% of the cohort was genotyped) and attrition may have accounted for the lack of a significant association. Various factors associated with phenotype/genotype discordance, such as advanced HIV illness, may have also contributed (see Section 2.3.3.1.1). The trend was not evident when the origin of the survival analysis was set at the baseline assessment rather than at the virtual time point of anti-TB therapy initiation. Assessing survival at the onset of exposure to an acetylation-dependent agent is intuitively the more correct analysis, even with the possibility of subject-recall bias (see Section 5.1.8).

The trend evident in the “left-shifted” analysis, although not significant, is made relevant when viewed in the context of the previously established biological association between slow acetylation and the risk for INH-PN. If the trend is accepted to have biological relevance, even if not statistically significant, then it may also be cautiously deduced that the risk for DSP in HIV/TB is attributable to INH, directly or indirectly. If we accept this assumption, we would then expect to find an associated B6 deficiency (B6 deficiency being the primary mechanism of INH-PN); however, deficiency was not demonstrated in the cohort. There are several possible explanations for an association with slow acetylation in the face of normal B6 status. First, deficiency prior to study entry cannot be excluded and deficiency at this time may have been contributory. Second, mechanisms other than B6 deficiency account for INH neurotoxicity (see Section 2.3.2.1.3) – levels of the INH by-product, hydrazine, are higher in slow acetylators[131]; hydrazine induces oxidative stress, an important element of ATN. However, Third, INH may induce a functional B6 deficiency even when supplementation corrects blood levels[26] (see Section 2.3.2.1.2). Finally, slow acetylation may represent an independent predictor of HIV/TB-associated DSP by unknown mechanisms. In one study, acetylation phenotype was implicated as a risk factor for diabetic

neuropathy because a greater proportion of DSP-free subjects were fast acetylators[192]; however; the association was later attributed to confounding – fast acetylators are over-represented in the type 1 diabetic population (possibly because of a hyperglycaemia-induced abundance of acetyl-CoA) and type 1 diabetics experience less neuropathy than type 2 diabetics[125].

Although fast/intermediate acetylators did not demonstrate higher PLP levels than slow acetylators, they did show greater increases in PLP levels from baseline to follow-up when compared to slow acetylators. Differences in B6 status between acetylation phenotypes have been demonstrated for the indirect GOT index only, and not for direct B6 assays[43,68]. The increase in PLP levels in fast/intermediate acetylators demonstrated here is consistent with other study findings – at follow-up, PLP levels were a function of INH dose (see Section 5.1.5), and presumably INH concentration, a function of acetylation status[26,68]. Lesser improvements in PLP levels may explain the trend for slow acetylators to develop DSP, although biologically, deficiency would be expected to be driving the risk.

Acetylation phenotyping was not performed in this study; discordance between predicted phenotype and true phenotype cannot be ruled out and may have affected associations observed[47-49]. Co-administered NAT2 substrates such as co-trimoxazole[49] may plausibly have also affected the phenotype, as could have advanced HIV and TB disease (see Section 2.3.3.1.1).

5.1.8 Elements of the study design may have introduced bias while others were advantageous

Selection bias was introduced on admission to DPM – only 10% of all TB cases in South Africa are admitted to hospital[121] and the TB inpatient population is biased towards a patient profile of chronic illness, advanced untreated HIV disease, malnutrition, substance use, poor compliance history, low socio-economic status and multiple-episode TB history[193]. These factors may also represent potential confounders; many increase the risk for both B6

deficiency and DSP. Caution in generalising these results to the broader population should be exercised.

The DPM hospital population is highly selected specifically in terms of gender because DPM is a predominantly male hospital (see Section 3.2.1.2). One would expect the study population to have been similarly skewed, but the male:female ratio was in fact reversed. The discrepancy is readily explained: because the ratio of long-term to short-term beds at DPM is much higher for males than for females, the female "turnover" is much higher. Therefore, a higher proportion of admissions is female. According to hospital statistics, in February 2011, for example, admissions were 33:47 male:female. The ratio of "ill" to "ambulant" beds is also higher for females – a larger proportion of females at any one time point are ill patients. This bias may have accounted for the association of INH-PN with female gender (see Section 5.1.3). In the absence of a specific indicator of general clinical status (such as the Karnofsky score), I considered BMI, WHO stage, $CD4^+$ T cell count and haemoglobin as proxies for overall clinical condition: females had lower $CD4^+$ T cell counts and lower haemoglobin levels (see Section 4.2.1); however, the latter is expected in the general population. All risk factor analyses were adjusted for gender, as well as for age.

Selection bias may have also occurred on enrolment. Participation in the study was restricted to patients on first-line first episode or retreatment anti-TB therapy. Patients with multi-drug resistance and adverse reactions to anti-TB therapy, who were excluded, may be particularly at risk for other complications, such as DSP. DSP rates in the study may therefore have underestimated those in the greater HIV/TB population. The advantage was a "cleaner" cohort. A high proportion of patients approached for recruitment into the study (**Figure 4-1**) declined to participate, which may have further biased the sample. However, sampling bias was avoided because sampling was both systematic (all admissions were considered for eligibility) and continuous (save for a one month break in recruitment) throughout the study period.

During the pilot period, participants receiving cART at baseline were excluded. These criteria were relaxed thereafter, with the advantage that ART-use could be entered into the cross-sectional analysis. Furthermore, as a result of the change in national guidelines for cART programs in May 2010 (TDF replaced d4T as the first-line NRTI backbone), fewer participants were commencing d4T. There was therefore less scope to longitudinally assess the effect of d-drugs on DSP risk.

The initial pilot study data were included in most aspects of the final analysis. The main difference between the pilot and main study participants was that pilot participants, by definition, were either ART-naïve or had no recent exposure to ART. Pilot participants were therefore excluded from analysis of ART-related baseline characteristics, but were included in the multivariate analyses, because their exclusion would have been detrimental in terms of sample size (at the cost of potential bias).

The study did not have a control arm. While it may have been revealing to compare DSP rates in HIV-uninfected patients, that HIV infection increases the risk for DSP is already established (see Section 2.1.2). It was felt that inclusion of HIV-uninfected patients would dilute the effect of HIV and increase the sample size. Seen differently, the study was not investigating HIV as the exposure of interest – HIV defined the population under study (see Section 2.1.1).

One advantage to undertaking research at an inpatient facility such as DPM is that it accommodates a relatively “captive” population – systematic sampling would be difficult at a busy outpatient facility, and unavoidable loss-to-follow-up could be accounted for in the more controlled inpatient setting. Other advantages are the relative assurance of compliance to treatments and a reliable dosing schedule.

The study included patients with prevalent DSP. This allowed a cross-sectional component and an opportunity to study the natural history of HIV/TB-associated. We were also able to retrospectively observe DSP

incidence in those with DSP at baseline (see Section 3.6.2.2). As exposures were ongoing at the time of study entry, exclusion of baseline DSP may have resulted in a survival bias. Furthermore, a “clean” cohort may paradoxically have given us fewer data – because of the significant attrition, incident cases would not have been observed.

Most aspects of the study (study design in part; field work; administration; data management, capture and analysis; and molecular laboratory work) were executed personally. Direct involvement with all aspects of the study promoted consistency; strengthened internal validity and the fidelity of study findings; and facilitated contextualisation and interpretation of the data.

There was heterogeneity in terms of previous and ongoing exposures to factors potentially contributing to DSP – ART (including neurotoxic d4T), INH and pyridoxine. **Figure 4-3** illustrates this heterogeneity in the study population. Heterogeneity, particularly of a temporal nature, is expected in this population (see Section 2.1.1 and **Figure 2-2**). There was further heterogeneity in terms of previous TB episodes, site of TB infection, and co-morbidities.

In most cases anti-TB therapy was initiated at the referring facility and admission occurred well after this point (median of 16.5 days). This duration was also highly variable within the sample (Q1-Q3 11-39.5), and was dependent on multiple factors, such as the availability of beds or transport delays. ART exposure was also variable: some participants were already on long-term therapy, some had previous but ill-defined ART exposure and others had their therapy switched during the course of the study. Over a third of the cohort had no recall or record of having received pyridoxine; after admission all received pyridoxine, but at different dosages. Pyridoxine doses were also changed through the course of the study. Heterogeneity, particularly of a temporal nature, contributed to bias and confounding, precluded stratification, complicated the analysis and may have obscured true associations.

Selection criteria attempted to reduce heterogeneity – for example, all participants received INH. The left-shifted survival analysis was valuable in mitigating the potential bias and confounding associated with a variable period between anti-TB therapy initiation and the baseline assessment. Variables were also dichotomised to reduce heterogeneity: use of ART was grouped into exposure ever, previous TB grouped into yes or no and pyridoxine standard or high dose.

The sample size was designed to detect baseline DSP. Loss-to-follow-up precluded reliable longitudinal analyses and so the power of the cross-sectional analysis was maximised. One participant was excluded after recruitment was ended; the sample is therefore one short of the intended size (116 vs. 117 participants).

5.1.9 Defining and measuring DSP is problematic

As expounded in detail in Section 2.2.4.1, there are caveats to the definition and diagnosis of DSP. We used the BPNS and a modified TNS to assess neuropathy status, in line with much of the current literature around HIV-associated DSP (see Section 2.2.4.3). Use of a modified TNS was justified for practical and cost reasons – neither nerve conduction study nor quantitative sensory testing facilities are available at DPM. A modified TNS has now been used in several studies[11,63,95,102], and the current trend is towards purely clinical DSP screening[35]. With respect to the BPNS, the TNS adds the important pinprick modality for assessing small-fibre function, and thus increases specificity for the diagnosis of HIV-associated DSP. Additionally, the TNS adds a score for toe/ankle muscle strength – distal weakness is a late feature of HIV-DSP, ATN and INH-PN (see Section 2.2.1) – prominent weakness would also alert the examiner to the possibility of other pathology not in keeping with a diagnosis of DSP.

The case definition of DSP in this study was ≥1 neuropathic symptom and ≥1 neuropathic sign. The relevance of one versus two signs for the definition of DSP has been debated[13] but the former is widespread in the literature (see Section 2.2.4.1). The two signs definition is considered conservative[11] and

lacking in sensitivity[77]. A significant caveat in this study is that the DSP-free comparator group comprised all participants not satisfying full criteria for DSP; therefore, by definition, participants with ADSP or with isolated neuropathic symptoms were included. There were multiple possible outcomes and comparators: for example, DSP vs. non-DSP; DSP vs. completely normal; ADSP vs. non-ADSP; or any symptoms vs. no symptoms. The decision to define the outcome as DSP vs. non-DSP was based on the following: the low proportion of participants who were entirely free of neuropathic symptoms and/or signs at baseline (only 15%); the lack of evidence supporting a distinct relationship between symptomatic DSP and ADSP (the significance of ADSP is debated, as is the significance of isolated symptoms [see Section 2.2.5.2]); and a focus on the more clinically relevant symptomatic DSP. A high prevalence of patients not satisfying DSP criteria but with neuropathic symptoms or signs alone is not unique to this study: in Wadley et al. this group comprised 25% of the cohort[12].

The definition of DSP severity and worsening (increased severity over time) was also problematic – worsening has not been uniformly defined in the literature, which focuses on DSP incidence rather than natural history (see Section 2.2.5.5). The TNS is not felt to be valid for assessing severity in a meaningful sense[34]; nevertheless, changing TNS score has been utilised in some studies[77,100], but the exact point-change equating to clinically meaningful change is essentially a guesstimate. I utilised a 2-point change because it would translate to either an improvement in two modalities, or a significant improvement in one single modality. However, a change in the score for symptoms could theoretically be balanced by an improvement in the score for signs, thereby obscuring a clinically-relevant worsening. For this reason I utilised an additional and separate definition of a 2-point change in either of the positive symptom NRSs (pain or paraesthesia). A 2-point improvement in pain NRS has been validated against a PGIC[98], but worsening has not received the same treatment. My definition is therefore extrapolated, but may have constructional validity. One further caveat was the treatment of the composite TNS score, a categorical variable by definition, as

a continuous variable in the analysis; however, scoring systems are often treated as continuous variables[11,63,77,98,194].

5.1.10 There were some methodological questions

The BPNS defines ankle reflex responsiveness relative to the knees, and failure to appreciate this relation may have affected inter-rater reliability in one study[77]. A study of acceptability of the BPNS for clinical purposes found accurate assessment of reflexes to be challenging to healthcare workers[97]. In the current study, emphasis was placed on discerning the ankle reflexes with reference to the knee reflexes, which may have reduced the false-positive rate, and may also account for differences in observed DSP rates in this study and others (see Section 5.1.1).

BPNS vibration scoring was hampered by ambiguous instructions. In the situation where a slight difference (1 or 2 seconds) between right and left toe vibration thresholds straddled the “normal” value of >10 seconds (e.g. 10 and 11 seconds), the BPNS instruction to “take highest score but both right and left must be abnormal” was ambiguous: the highest score (i.e. the worse threshold) is interpreted as abnormal but both scores were not abnormal, precluding classification. This situation occurred in 16% of assessments. Our decision was to classify vibration sense as normal – the scores were sufficiently close to normal, and the BPNS specification that both reflexes should be abnormal was adhered to.

The BPNS specifies activating the tuning fork by squeezing the two ends of the fork together followed by a sudden release, in contrast to striking the fork on a hard surface. My feeling is that squeezing affords a more consistent intensity of vibration – a constant set force is required to oppose the two ends of the fork (potential energy) whereas a striking force is dependent on velocity (momentum) and the surface being struck. Squeezing versus striking may very well affect observed DSP frequency and incidence as several seconds difference can alter the outcome. While inter-rater variability in vibration technique was not a major issue in this study – the vast majority of

assessments being performed by myself – it could very well influence validity when comparing and generalising study findings.

Another debatable point regarding vibration threshold testing arose when my supervisor attended a demonstration of the BPNS for clinical trial purposes (by David Simpson, Pfizer) in which it was stipulated that vibration thresholds should be timed from the point of activation of the fork, rather than from the point of application to the target area. This was in disagreement with the method we were utilising in this study and as specified in the BPNS: "immediately place the vibrating tuning fork on the [area] and begin counting the seconds". An argument for "pre-timing" is that the vibration intensity decays uniformly after activation and it is this absolute intensity that is perceived by the subject: the subject will perceive vibration until a certain intensity threshold is reached irrespective of when the vibrating fork is applied. However, this method presupposes that the rate of decay of vibration intensity is uniform pre- and post-application. If the rate of decay changes when the fork is applied to the skin, then the period of non-contact will bias the recorded threshold and introduce unaccounted for variability. After discussion of these various points, we felt that since we were timing sensation, only the time spent actively sensing the vibration should be timed. As the study was well underway, the point was moot, and consistency in the technique was considered the more important factor.

It was my subjective impression over the course of the study that vibration thresholds were reduced by cold skin and/or ambient temperature – the influence of cold ambient temperature on sensory modalities other than vibration have been demonstrated[195], but data on vibration sense is lacking. An interesting approach would have been to measure skin and ambient temperatures at each visit and correlate temperatures with vibration thresholds, or to do a sub-study in which thresholds are assessed before and after limb cooling.

5.1.11 Analysis of longitudinal data presented challenges

Attrition was a major biasing element of the study and complicated analysis. The high attrition rate was a result of the inpatient nature of the study – involvement with the study was during admission only, and participants were not followed-up post-discharge for practical and cost reasons. Loss-to-follow-up was due to multiple factors (**Figure 4-2**) which were on the most part unavoidable – the development of acute illness and toxicity; patient transfer; self-discharge; and death could not be predicted or controlled. Patient discharge was an exception: as discharge could be anticipated in advance, there was a possibility of follow-up just prior. Follow-up occurred if two weeks had elapsed since the previous assessment, but occasionally participants due for discharge were missed. Participants lost to follow-up before the eight week assessment differed from those who survived to this point – they had higher BMIs, possibly because the majority of losses by eight weeks were due to discharge. The remaining cohort may have biased by participants who were sicker. Biases related to loss-to-follow-up may have influenced study findings, and because loss-to-follow-up in this study was not random, it is difficult to adjust for[196].

Incident and worsening DSP were considered separately – analysis of combined incident/worsening would be complex in the context of a survival analysis wherein the two outcomes are semi-competing. Furthermore, the population at risk for incident DSP and that at risk for worsening DSP are mutually exclusive. Worsening DSP is also a poorly understood entity (see Section 2.2.5.2) and may be a heterogeneous process not equivalent to incident DSP. The lack of independent associations with worsening DSP in this is supportive of this hypothesis.

A result of separating the analysis for incident and worsening DSP was that the sample entered into the survival analysis for DSP incidence was reduced (only those DSP-free at baseline were eligible). This issue was partially mitigated by the left-shifted survival analysis wherein patients without follow-up data were included (see Section 3.6.2.2). Further long-term research and

larger sample sizes are required to further delineate incident and worsening DSP, and associated risk factors in HIV/TB populations.

5.1.12 Molecular techniques required some troubleshooting

The molecular component of the project involved a steep learning curve, a process characterised by a number of obstacles. Initially, DNA extractions yielded low DNA concentrations and had to be repeated for several samples. Troubleshooting involved replacement of several reagents, extending incubation times and increasing the number of washes. The latter was not fruitful, reducing concentrations rather than increasing them. Further refinement of the technique eventually produced better results. Samples of concentration <50 but >20 ng/µml were also successfully amplified by PCR.

Restriction enzyme digestion with *KpnI* and *MspI* (digest "A") produced an anomalous ~800 bp fragment in two samples (**Figure 4-9**, lane 5). A fragment of this size would suggest the $G^{191}A$ and $C^{481}T$ SNPs occurred on the same chromosome; although, if this were the case, the remaining bands would imply three alleles. The ~800 bp fragment could not be explained by incomplete *MspI* digestion because the 96 bp fragment was present – the ~800 bp fragment would not be possible if *MspI* was inactive. Digestions with each of *KpnI* and *MspI* were run separately in which anomalous fragments were also demonstrated. Contamination would account for the presence of three possible alleles, but repeat extractions and digestions continued to demonstrate the anomalous fragments. Blood transfusion is a plausible explanation for apparent contamination of samples collected at different time points, but there was no history of a transfusion. To produce the ~800 bp fragment there must have been decreased *KpnI* activity at the 481 locus, possibly due to some unidentified genetic or extrinsic factor. Non-specific amplification of *NAT1* by the *NAT2* primers is another possible explanation. Sequencing eventually confirmed heterozygosity for alleles at each of the 191 and 481 loci (**Figure 4-13**).

The allele-specific PCR for the determination of the $T^{341}C$ SNP proved to be unreliable, necessitating confirmatory sequencing for all heterozygotes. The

validity of allele-specific PCR is intrinsically compromised by the employment of two separate reactions, effectively doubling the chance of error[122]. The technique has received further criticism in the literature: O'Neil et al. reported "high background"[48], while Doll et al. suggests an alternative nested PCR restriction enzyme digestion[181]. Our results further support these observations.

5.1.13 Ethical issues were balanced on the whole

Potential harms in the study were few and involved the risks and discomfort of venesection; the effort expended to complete the assessments; and the potential for breech of confidentiality (for example, having one's HIV status inadvertently revealed because one is known to be participating in the study). Potential harm was avoided in the following ways: patients that were considered too ill to participate in the study were not enrolled; a private consulting room was used; study materials, samples and data were anonymised; and no more than two attempts at venesection were made. Participants were protected by the approval of an accredited ethics committee, and the informed consent document.

The patient population at DPM may be considered disenfranchised and vulnerable, and some patients might not have necessarily appreciated the voluntary nature of research participation. Voluntary participation was therefore stressed, which is evidenced by the number of patients declining participation and opting out of the study (see Sections 4.1). (These factors may have introduced bias, however [see Section 5.1.8].) Participants did not receive any financial compensation for partaking in the study, as they did not incur transport costs or potential loss of income. Some patients with documented HIV infection were not aware of their HIV status or were in denial about their status, or had not received adequate post-test counselling. They were not enrolled into the study so as not to confront them with these issues during the study process. Participants who did not fully comprehend the informed consent process were not enrolled.

There were minor benefits to the participants themselves: uncovering of undiagnosed DSP, ready access to neurologist advice/consultation if required and an additional line of communication between the patient and DPM medical staff. A line was drawn, however, between the research process and treating/advising the patient[197]. Harms were further balanced indirectly by potential contribution to science and benefits to the greater HIV/TB population – HIV/TB-associated DSP is a neglected topic, and management avenues are limited. Patients may benefit from this research in the future.

5.2 Contributions and recommendations

This study represents the first detailed description and analysis of HIV/TB-associated DSP – current literature is confined to one short report[2], and to studies of general adverse events in HIV/TB (see Section 2.1.2). Study findings have already been presented as a poster presentation at the Neurological Association of South Africa Congress 2012. A paper intended for publication in an international peer-reviewed journal is currently being drafted.

Despite a high level of care (relative to an outpatient clinic setting), DSP was under-recognised and under-treated. This situation is not unique to this hospital (see Section 2.4.1.1), possibly because DSP assessments were not routine relying on patient-reported symptoms. This situation is a product of over-stretched resource-limited clinical services – more serious medical issues will be prioritised in these settings. Our research serves to highlight the significant morbidity associated with HIV/TB-associated DSP in the form of painful and other symptoms, and the high prevalence and incidence of this complication. Neuropathic pain is distressing and disabling and should therefore not be disregarded or left untreated. Greater sensitivity in diagnosis and closer monitoring of DSP may address this issue. The NRS and PGIC scales are simple tools for gauging objective changes in symptom severity that may be used clinically to guide therapy. Similarly, a screening tool such as the BPNS could easily be incorporated into routine assessments[97]. However, a tuning fork and patellar hammer are necessary, and assessments can be time-consuming at times, particularly when reflexes are difficult to elicit. These limitations are relevant to resource-limited settings. Furthermore,

the most common neuropathic sign associated with DSP in this study was impaired pinprick sensation, suggesting it is the most sensitive modality. Assessment of pinprick sensation can be rapidly performed and requires only a sharp object, but is not tested in the BPNS. A caveat in resource-limited settings with a high prevalence of infectious disease, such as hepatitis B, is the danger of cross-infection when pins or other sharp objects are reused. History-based screening for DSP may also have utility (see Section 2.2.4.4) – in this study only six participants had symptoms in the absence of signs, two of whom subsequently developed full DSP.

In this study, under two thirds of the cohort received pyridoxine supplementation at the referral facility prior to transfer to DPM; this figure is lower in an exclusively outpatient setting[11]. There are several possible reasons: supply-chain shortages; deficient guidelines; underestimation of the risks for and clinical impact of DSP; and an unfounded fear of precipitating a pyridoxine neuropathy. The literature adequately demonstrates increased risk for B6 deficiency in HIV/TB populations; our research shows that pyridoxine 25 mg/day is probably sufficient to prevent or reverse this expected deficiency. Pyridoxine should therefore not be withheld in this population, and a similar emphasis on compliance afforded to other HIV/TB therapy should be placed (e.g. directly observed therapy and pill counts).

Higher dose pyridoxine (>25 mg/day) may be unnecessary for the purpose of preventing or treating B6 deficiency. Furthermore, the practice of prescribing higher dose B6 for the treatment of established INH-PN is not evidenced, and an additional neuroprotective benefit of higher dose pyridoxine cannot be inferred in the context of adequate B6 status. It could be argued, however, that, in the absence of concrete evidence supporting a lack of benefit, no specific harm may result from pyridoxine supplementation in any dose not exceeding safe limits. The implications of unnecessary higher dose supplementation may be subtle: while pyridoxine is not an expensive agent (on the order of <US$0.1/25 mg), over-prescription in resource-limited settings may result in shortages; a perceived therapeutic benefit may result in a failure to treat DSP symptomatically; and emphasis may be shifted from prophylaxis

to therapy. An increased pill burden may impact on adherence. Pyridoxine should be supplemented at standard doses of 25 mg/day in the absence of evidence showing a benefit at higher dosages.

While B6 deficiency may be prevented or corrected by pyridoxine supplementation, we either do not have enough information to show that it is neuroprotective in this population, or, if we accept the current evidence, the mechanism of neurological injury is not entirely B6-related. However, there remains a possibility that DSP rates may be even higher without supplementation or that prior deficiency may still be contributory. Our data did not associate the absence of pyridoxine prior to admission with prevalent or incident DSP – poor reliability of this historical data may account for the lack of association. As deficiency is correctable, and is an established risk factor for INH-PN, again, we recommend consistent supplementation from the time of anti-TB therapy initiation. Because vitamin B-complex, widely administered in HIV, is not sufficient to prevent B6 deficiency[112], an argument can be made for instituting full B6 supplementation even before anti-TB therapy.

Our research adds to the knowledge base for *NAT2* genetic variation and acetylation phenotypes in our local populations. There are suggestions in the literature that acetylation status guide INH dosing schedules[29,45]. The trend for slow acetylators to develop DSP in this study is not sufficient to promote determination of acetylation status prior to anti-TB therapy prescribing in HIV/TB co-infection, but also does not exclude a potential benefit of this approach. However, the practicality of instituting widespread phenotyping or genotyping to determine acetylation status is doubtful in our setting.

5.3 Further research

The current dataset and sample bank is amenable to further study. I would prioritise completion of *NAT2* genetic analysis for the entire cohort; a further 33 participants may be typed. Although these participants have no follow-up data, they may still be entered into the cross-sectional and “left-shifted” analyses. The increased sample size may strengthen statistical significance.

However, because of uncertainty around B6 sampling, I do not feel it worthwhile to extend B6 analysis to the rest of the cohort.

The longitudinal data, although incomplete, may benefit from further analysis. General estimating equations, for example, are useful for the analysis of unbalanced longitudinal datasets[176], and for the analysis of panel data (data viewed as sequential cross-sections) in which the outcome may be present or absent at each time point. A multiple failure survival analysis may also be worthwhile in this respect – the benefit would then be that failure does not equate to data censoring. Only baseline covariates were examined, but several were also time-varying and should be entered into future analyses. Other outcomes may also be considered, such as ADSP and improvement of DSP (as opposed to worsening), and the cohort stratified by aetiological diagnoses.

As plasma and serum samples are available for all participants at all time points, we explored the possibility of running INH assays and using the INH metabolic ratio to establish acetylation phenotype. The INH plasma concentration curve is highly variable and accurate pharmacokinetic assessment requires serial sampling[121]. Plasma was sampled at only one time point, however, and too great an interval post- dosing. Furthermore, a 3-hour sample is required for accurate phenotyping[119]. Other possibilities for laboratory analysis include mitochondrial DNA haplotyping and markers of oxidative stress and/or inflammation.

Heterogeneity and incomplete follow-up are factors detracting from further study of the dataset. Ethics are in the balance: unused samples and data versus expending further resources on a limited project. The decision to abandon or continue study of the data does not necessarily need to be made immediately; useful genetic or biochemical markers may be discovered in the future.

The design of future studies should be guided by the lessons learned in this study. Patients would ideally be enrolled at the onset of TB symptoms and

followed for the duration of anti-TB therapy, and after. An outpatient setting would be suitable for this study design, but may select out well patients. A very well-resourced study would allow follow-up of patients as they are transferred from facility to facility. Vitamin B6 levels should be sampled prior to commencing therapy, and then pre-dosing. More detailed information regarding previous medical history would better elucidate possible previous neurological insults. The sample size should accommodate stratification of participants by ART exposure. Genotyping could be confirmed by appropriate phenotyping. The study of HIV/TB-associated DSP requires long-term follow-up of HIV-infected patients; the feasibility of this kind of research should be carefully considered.

One of the study aims was to inform the design of an RCT that would determine optimum pyridoxine dosing in this HIV/TB population. We have established that standard doses will prevent deficiency, but not DSP. An RCT may then be justifiable to test the hypothesis that higher dose pyridoxine is beneficial neurologically despite the correction of B6 deficiency. As there is no equipoise regarding the benefit of supplementation (see Section 2.4.3), a placebo arm would not be justifiable.

5.4 Conclusion

Symptomatic DSP is frequent in HIV/TB co-infected patients recently commenced on first line anti-TB therapy, and continues to develop or worsen thereafter at a high rate. However, over time, the overall trend is for symptomatic improvement. HIV-DSP, ATN and INH-PN all contribute to DSP in HIV/TB co-infection, but separating aetiological processes may require earlier and more prolonged longitudinal study. A possible “TB-DSP” is an entity that may have clinical implications, and also requires further investigation. The clinically significant symptoms apparent in this study were under recognised and under-treated – screening for DSP should be part of the routine assessment of the HIV/TB co-infected patient. Symptom only screening may be appropriate in busy resource-limited settings.

The clinical risk profile for DSP supports the prevailing theory that DSP is a multi-factorial process, and that DSP is a function of cumulative neurological

insults. Prevalent DSP was associated with previous ART use, and incident DSP with concomitant ART; however, the associations could be explained by other demographic and disease factors in multivariate analysis. Previous TB, implying INH exposure, was independently associated with INH-PN. More prolonged and consistent follow-up would strengthen risk association findings.

DSP was not found to be associated with vitamin B6 deficiency in this study; the lack of association is because patients receiving ≥25 mg/day pyridoxine supplemented anti-TB therapy do not demonstrate B6 deficiency. Nevertheless, adequate B6 status, as estimated several time intervals after the initiation of pyridoxine supplementation, does not appear to prevent incident or worsening DSP. Pyridoxine doses in excess of the standard 25 mg/day, prescribed in response to the diagnosis of DSP, did not improve B6 status as estimated by plasma PLP – the excess pyridoxine is probably metabolised to 4PA once steady-state is achieved. Study findings relating to B6 are probably not sufficiently reliable to conclusively inform the design of an RCT or to make definitive recommendations, mainly because of methodological questions around B6 sampling and dosing.

Slow acetylation was not a primary risk factor for DSP in this population; however, there was a trend for slow acetylators to develop DSP when compared with fast/intermediate acetylators. Slow acetylation did not show an association with B6 deficiency, because deficiency was almost completely absent from the sample. The trend for slow acetylators to develop DSP is therefore not likely to be explained by this mechanism. Direct INH toxicity in the form of oxidative stress may be responsible, possibly exacerbating oxidative damage related to inflammation and d-drugs. A contribution from B6 deficiency prior to sampling cannot be excluded and again, earlier sampling is advisable in future studies. NAT2 phenotyping would also help corroborate these findings.

The primary (alternative) hypothesis is rejected: in HIV/TB co-infection, DSP is associated with neither vitamin B6 deficiency, nor slow acetylation status.

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Appendix 1

Information sheet and informed consent document

Patient Information Sheet for the Neuropathy Study

Title: **Isoniazid-TB treatment as risk factor for HIV-associated Neuropathy and the role of adequate B6 supplementation**

Dr C Centner & A/Prof J Heckmann (Neurology); University of Cape Town

You are invited to participate in a research project at DP Marais Hospital. Before you agree to take part you need to understand the following:

One of the possible complications of HIV infection could be damage to the long nerves supplying the skin of the feet and legs causing most commonly pain and tingling in the feet. This problem is called peripheral neuropathy. Unfortunately, it may also be a side effect of the treatment used to treat HIV, highly active antiretroviral therapy; or HAART for short (also known as ARVs - anti-retrovirals). This is a combination of special medications to try and stop the HIV from dividing in the cells of the body and causing harm. Although HAART/ARVs cannot cure HIV/AIDS (i.e. some virus still stays in the cells) it has helped to fight the development of AIDS and has helped and saved a lot of patients. So, although HAART/ARVs is useful in treating HIV, it may also cause or worsen peripheral neuropathy in some patients and very little is known about this problem in South African patients. In this study we want to learn more about why some patients with HIV and those taking HAART/ARVs develop painful feet, and how treatment for TB and vitamin levels in the body (vitamin B6 especially) can contribute to this problem. This will help us to develop ways to prevent or treat this side effect of HAART/ARVs.

It is important to realise that not everyone on HAART/ARVs will develop peripheral neuropathy, but some individuals are at risk of developing this side effect. It will be useful to identify who are at-risk so that we can try and prevent this complication from developing.

WHY HAVE I BEEN CHOSEN?

We are discussing this project with you because you are HIV-infected which will mean that some point in the near future you will start on ARV treatment. Also, you have tuberculosis which means that you are already receiving TB treatments that includes INH, for at least 6 months. INH by itself can result in peripheral neuropathy in certain individuals who don't have other risk factors such as HIV or ARV therapy. Additional vitamin B6 treatment usually helps this symptom of painful feet. With this study we want to determine if all subjects with HIV/AIDS and TB require the same amount of vitamin B6 to prevent peripheral neuropathy?

From your hospital records we will know how much vitamin B6 you are taking. We want to study the vitamin B6 levels in the blood and see whether you have enough for your body's needs. We also want to take blood for a genetic test called NAT2 which we think, may tell us whether a person's body needs much more vitamin B6 or not. The NAT2 gene is important in inactivating the INH-TB treatment. We think half the general population are "slow metabolizers or inactivators" as a result of their NAT2 gene arrangement and that this may affect the individual's need for vitamin B6. "Slow INH inactivators" may need more B6 than NAT2 "fast inactivators"

WHAT WILL HAPPEN IF I AGREE TO PARTICIPATE?

The investigating doctor will assess you soon after your admitssion to DP Marais hospital and again after 1 and 2 months while you are still a patient there. The assessment will include a short interview about symptoms you are having, a short examination of the feet and legs as well as blood drawing (not more than 15-20 mls / 3-4 teaspoons of blood will be taken). The blood will be sent to the laboratory for vitamin B6 and DNA genetic testing (NAT2). You will need to provide separate permission for the DNA genetic testing. Some of the blood will be frozen and kept for testing at a later stage. Stored blood and genetic samples will not be labeled with your personal information.

The study staff will also review all of your previous clinic records including all of your previous blood and other test results (including HIV status, viral load, CD4 count, liver function, kidney function).

WHAT ARE THE POSSIBLE DISCOMFORTS OF PARTICIPATING IN THE STUDY?

Having blood taken will be the only discomfort in this study. Risk of infection will be minimized by using sterile procedures, and all blood samples will be taken by a qualified study doctor.

WHAT ARE THE POSSIBLE BENEFITS?

If you are found to have complicated HIV-related nerve problems then arrangements will be made for you to be seen at Groote Schuur Hospital where doctors specially trained in looking after these problems will assess you. More generally, you will be contributing to our understanding of peripheral neuropathy in HIV and it's treatment, and other patients in the future will benefit from this.

DO I HAVE TO PARTICIPATE?

You do not have to participate in this study. Your participation is voluntary and if you agree to participate then you will be required to sign a form. You can withdraw from the study at any time and this will in no way affect your treatment in the future.

WILL THE INFORMATION REMAIN CONFIDENTIAL?

Your records will only be viewed by your doctors and people involved in this study, including research oversight agencies, which support the rights of subjects involved in research (such as ethics committees or the Office of Human Research Protections). Your details will not be made available to anybody not involved in this study. Although absolute confidentiality cannot be guaranteed, the staff involved in this study will strive to keep your records as confidential as possible.

CONTACT DETAILS OF STUDY STAFF

Should you have any questions, then please contact the study doctor Dr Chad Centner on 082-253-7618 (via call, SMS or please-call-me) or leave a message at UCT neurology 021-404-3198. The UCT Human Research Ethics Committee may be contacted at 021-406-6338.

UCT ICD DP Marais modified 15 Feb 10

Written Informed Consent for the Patient

Study Number: ..... Patient Initials: .....

I,.....
(Name of patient in block letters)

Have read and understood all the information given to me about my participation in this study and I have been given the opportunity to discuss it and ask questions. I voluntarily agree to take part in this study and have received an information sheet outlining the details of this study. I understand that I am able to withdraw from this study at any time.

Please tick the boxes if you are willing to give a 20mls (4 teaspoons) of your blood for storage to be used by this study and /or at a later date; any further research studies must and will always first be cleared by the UCT research ethics committee before the researchers can use your blood.

- I am willing to allow a blood specimen to be taken for vitamin B6 levels: YES NO.

- I am willing to allow a blood specimen for DNA extraction to determine my NAT2 gene status: YES NO.

- I am willing to allow the left-over DNA to be used for possible future genetic studies IF the research project has been cleared by the UCT research ethics committee before the researchers can use my specimen YES NO.

- I am willing to allow the left-over blood to be used for possible future studies IF the research project has been cleared by the UCT research ethics committee before the researchers can use my specimen YES NO

Signature of patient....., Date .....

.....
Printed Name of Patient

I have explained the nature and purpose of the study to the patient named above.

.....	.....	.....
Signature of doctor	Print Name of Doctor	Date

Appendix 2

Baseline and follow-up data collection forms

HIV-Associated PN Study at DP Marais Patient Data Capture Sheet

NO:	T	BASELINE	Date:	dd	mm	yy

Only initials to be entered into study database				Ward:	
Name:				Date admitted:	
DOB:	dd	mm	yy	dd	mm	yy
F/N:				Height:	
			Affix label here	Weight:	

PMHx	DM	Epilepsy	Renal dysfunction	Pregnancy
	Previous TB:	yy	yy	yy	Other:

ETOH	When was the last time you had more than 4/5* drinks in 1 day?
	Never >1 year ago <1 year ago

TB	Site	Confirmation	Regimen	Current dose
	PTB Other:	AFB x1 x2 x3 Culture + - pend Radiological Other:	1 2	RHZE: RH: Strep:
	Starting date:	dd mm yy	**Interruptions:**	days

HIV	Tested:	dd	mm	yy	Stage:		CD4C:	
	Current ARV regimen							
	nil	D4T:		AZT:		TDF:		
		3TC:						
		EFV:		NVP:		Other:		
	Starting date:	dd	mm	yy	**Interruptions:**		days	
	Previous ARV exposure							
	nil	D4T	AZT	TDF	3TC	EFV	NVP	Unkown
	Last taken:	dd	mm	yy	**Time on Rx:**		months	

Neuro	Dx of PN		Treatment		
	Yes	No	Amytriptylline:		Other:
			Start:	dd mm yy	

B6	Pyridoxine-HCl	Start:	dd	mm	yy	Dose:	
		Change:	dd	mm	yy	Dose:	
		Change:	dd	mm	yy	Dose:	
	Levels taken?	Y N	Hours since last dose:				
	Vit Bco	nil	i	ii			

Rx	Bactrim:	Phenytoin:	Other:

Bloods	Date taken:	dd	mm	yy				
U:	Cr:	Alb:	ALT:	AST:	WCC:	Hb:	MCV:	Plt:	HepB:

*4 for women, 5 for men

HIV-Associated PN Study at DP Marais Patient Data Capture Sheet

NO:	T	FOLLOW-UP	Date:	dd	mm	yy

Ward:		Weight:	

TB	TB culture + - pend
	Toxicity:

HIV	ARV regimen							
	nil	D4T:		AZT:		TDF:		
		3TC:						
		EFV:		NVP:		Other:		
	Start:	dd	mm	yy		Interruptions:	days	
	Toxicity:							

Neuro	Dx of PN		Treatment		
	Yes	No	Amytriptyline:		Other:
			Start:	dd	mm	yy

B6	Pyridoxine-HCl		Change:	dd	mm	yy	Dose:	
			Change:	dd	mm	yy	Dose:	
		Levels taken?		Y N	Hours since last dose:			
	Vit Bco		nil	i	ii			

New Dx	

New Rx	

Bloods	Date taken:		dd	mm	yy			
U:	Cr:	Alb:	ALT:	AST:	WCC:	Hb:	MCV:	Plt:

Appendix 3

Baseline and follow-up neuropathy capture forms

Revision V3 March 2010

SA BRIEF PERIPHERAL NEUROPATHY SCREENING/EXAM (UCT REC 221/2008)

NO:	**T**	**BASELINE**	**Date:**	dd	mm	yy

We are going to ask you a few questions about sensation in your legs. We will also briefly examine the nerves in your arms and legs. We may advise the clinic doctor on treatment.

BPNS : INSTRUCTIONS FOR RECORDING SYMPTOMS: *Ask subject to rate the severity of each symptom in 1a to 1c on a scale of 0 (absent) to 10 (most severe) for right and left feet, legs- worst in last week*. Enter the score for each symptom in the block marked Severity. Enter extent of symptoms eg Soles of feet/ toes (TNS=1); up to ankle (TNS=2); up to knee (TNS= 3) or above (TNS=4) on the TNS score overleaf.

1a. Pain, aching, burning in feet or legs. **Ingaba iinyawo zakho zibuhlungu, ziyaqagamba, ziyatshisa kangangee-veki ezimbini?**

Normal	Mild	→	→	→	→	→	→	→	→	Severe
0	1	2	3	4	5	6	7	8	9	10
Andinantlungu! →			→		→		→		**Ndineentlungu ezigqithisileyo!**	

Nn1 score 1a	

1b. "Pins-and-Needles" in feet or legs. **Ingaba iinhawo zakho zineenaliti noonotaka kangangee-veki ezimbini?**

Normal	Mild	→	→	→	→	→	→	→	→	Severe
0	1	2	3	4	5	6	7	8	9	**10**
Andinantlungu! →			→		→		→		**Ndineentlungu ezigqithisileyo!**	

Nn2 score 1b	

1c. Numbness (lack of feeling) in feet or legs. **Ingaba iinyawo zakho zinobundindisholo kangangee-veki ezimbini?**

Normal	Mild	→	→	→	→	→	→	→	→	Severe
0	1	2	3	4	5	6	7	8	9	**10**
Andinantlungu! →			→		→		→		**Ndineentlungu ezigqithisileyo!**	

Nn3 score 1c	

TOTAL SENSORY PRESENCE/SEVERITY SCORE: Obtain the single highest severity score from 1-10 in 1(a - c) above:

0 = Grade 0 **1-3 = Grade 1** **4- 6 = Grade 2** **7-8 = Grade 3** **9-10 = Grade 4**

Total sensory severity GRADE ____/4 nn4

Patient asymptomatic (1a,b,c = 0) □

1d. Has patient had any symptoms in the past which are not currently present? Yes (1) No (0) nn5

Patient symptomatic □

1e. Relationship of onset of symptoms to current TB symptoms and regimen?

Before TB Sxs (1) After TB Sxs but before TB Rx (2) After TB Rx (3) Unsure (4) nn27

1f. Relationship of onset of symptoms to current ARV regimen?

Not on ARVs (1) Before ARVs (2) After ARVs (3) Unsure (4) nn28

1g. Any medication taken for the symptoms prior to admission at DPM? Yes (1) No (0) nn29

1h. If yes, what? _____nn30 How did the symptoms respond?

Much Worse (1) Worse (2) No change (3) Better (4) Much Better (5) nn31

1i. Does the patient experience leg cramps? Yes (1) No (0) nn6

2. INSTRUCTIONS FOR EVALUATING PERCEPTION OF VIBRATION:

Press the 2 ends together of a 128 Hz tuning fork, and release suddenly; place the vibrating tuning fork on the subject's clavicle; can they recognise the vibration or "buzzing" (ngcungcazela) of the tuning fork? Repeat and immediately place the vibrating tuning fork firmly on the interphalangeal bone (not nail) of one great toe and begin counting the seconds. Subject to tell you when the "buzzing" stops. Repeat on the other side.

Vibration Perception
(Take highest score but both R & L must be abnormal)

0- Vibration felt for >10 seconds (normal)
1- Vibration felt for 6-10 seconds (mild loss)
2- Vibration felt for 5 seconds or less (moderate loss)
3- No feeling of vibration (severe loss)

	Right	Left		
Great toe interphalangeal bone	____	____	(2) use highest value_____/3	nn11

3. INSTRUCTIONS FOR EVALUATING DEEP TENDON REFLEXES:

With the subject seated, the examiner uses one hand to press upward on the ball of the foot, dorsiflexing the subject's ankle to 90 degrees. Using a reflex hammer (long-handled), the examiner strikes the Achilles tendon.

Reflexes
4- Absent
3- Reduced (difficult to elicit)
2- Normal deep tendon reflexes
1- Hyperactive deep tendon reflexes
0- Clonus

	Right	Left		
Ankle Reflexes: (Take highest score but both R & L must be abnormal)	_____	_____	(3) use highest value____/4	nn12

Final score for BPNS (1+2+3) ____/11 nn13

Reduced TNS score: tick in the box :

	0	1	2	3	4	
a. Sensory symptoms from 1a,b,c: Pain, burning pins or numbness	*none*	*Only in toes or soles of feet*	*Symptoms extend to ankle or wrist*	*Symptoms extend to knee or elbow*	*Symptoms knee or elbow or functionally disabling*	nn14
b. Pin sensibility	*normal*	*Reduced in fingers /toes*	*Reduced up to wrist/ ankles*	*Reduced up to elbow/ knee*	*Reduced above elbow/ knee*	nn15
c. Vibration sensibility (use normal as for BPNS)	*normal*	*Reduced in fingers /toes*	*Reduced up to wrist/ ankles*	*Reduced up to elbow/ knee*	*Reduced above elbow/ knee*	nn16
d. Deep tendon jerks	normal	Ankle reflexes reduced	Ankle reflexes absent	Ankle reflexes absent, other reduced	All reflexes absent	nn17
e. Strength- ankle and toes plantar & dorsi-flexion	normal	Mild weakness (MRC 4)	Moderate weakness (MRC 3)	Severe weakness (MRC2)	Paralysis (MRC 0-1)	nn18

Final score for rTNS (a+b+c+d+e) _____/20 nn19

Neuropathy classification according to TNS:

Symptomatic DSP= a ≥1 and b≥1 or c≥1 or d≥1 or significant touch-evoked pain yes/ no _____ n20

Asymptomatic DSP= TNS ≥3 yes/ no _____ nn21

Indeterminate DSP= a ≥1 only yes/ no _____ nn22

Punch Biopsy performed yes/ no _____ nn23

Examination shows another form of peripheral neuropathy (not symmetric distal sensory) yes/no_____ nn24
refer for further evaluation to neuromuscular HIV clinic at GSH (1st wed am of every month; 404-3209)

Proprioception in both toes Normal=0 reduced=1 absent=2 _____ nn25

Revision V3 March 2010

SA BRIEF PERIPHERAL NEUROPATHY SCREENING/EXAM (UCT REC 221/2008)

NO:	**T**	**FOLLOWUP**	**Date:**	dd	mm	yy

We are going to ask you a few questions about sensation in your legs. We will also briefly examine the nerves in your arms and legs. We may advise the clinic doctor on treatment.

BPNS : INSTRUCTIONS FOR RECORDING SYMPTOMS: *Ask subject to rate the severity of each symptom in 1a to 1c on a scale of 0 (absent) to 10 (most severe) for right and left feet, legs- worst in last week*. Enter the score for each symptom in the block marked Severity. Enter extent of symptoms eg Soles of feet/ toes (TNS=1); up to ankle (TNS=2); up to knee (TNS= 3) or above (TNS=4) on the TNS score overleaf.

1a. Pain, aching, burning in feet or legs. **Ingaba iinyawo zakho zibuhlungu, ziyaqagamba, ziyatshisa kangangee-veki ezimbini?**

Normal	Mild	→	→	→	→	→	→	→	→	Severe
0	1	2	3	4	5	6	7	8	9	10
Andinantlungu! →			→		→		→		**Ndineentlungu ezigqithisileyo!**	

Nn1 score 1a	

1b. "Pins-and-Needles" in feet or legs. **Ingaba iinhawo zakho zineenaliti noonotaka kangangee-veki ezimbini?**

Normal	Mild	→	→	→	→	→	→	→	→	Severe
0	1	2	3	4	5	6	7	8	9	**10**
Andinantlungu! →			→		→			**Ndineentlungu ezigqithisileyo!**		

Nn2 score 1b	

1c. Numbness (lack of feeling) in feet or legs. **Ingaba iinyawo zakho zinobundindisholo kangangee-veki ezimbini?**

Normal	Mild	→	→	→	→	→	→	→	→	Severe
0	1	2	3	4	5	6	7	8	9	**10**
Andinantlungu! →			→		→			**Ndineentlungu ezigqithisileyo!**		

Nn3 score 1c	

TOTAL SENSORY PRESENCE/SEVERITY SCORE: Obtain the single highest severity score from 1-10 in 1(a - c) above:

0 = Grade 0 **1-3 = Grade 1** **4- 6 = Grade 2** **7-8 = Grade 3** **9-10 = Grade 4**

Total sensory severity GRADE ____/4 nn4

Patient is symptomatic for the first time since commencing study □

1d. Relationship of onset of symptoms to ARVs?

Before ARVs (1) After ARVs (2) Not on ARVs (3) Unsure (3) nn32

Patient had symptoms at previous visits □

1e. How have the symptoms changed since the last visit?

Much Worse (1) Worse (2) No change (3) Better (4) Much Better (5) nn33

1f. Does the patient experience leg cramps? Yes (1) No (0) nn6

2. INSTRUCTIONS FOR EVALUATING PERCEPTION OF VIBRATION:

Press the 2 ends together of a 128 Hz tuning fork, and release suddenly; place the vibrating tuning fork on the subject's clavicle; can they recognise the vibration or "buzzing" (ngcungcazela) of the tuning fork? Repeat and immediately place the vibrating tuning fork firmly on the interphalangeal bone (not nail) of one great toe and begin counting the seconds. Subject to tell you when the "buzzing" stops. Repeat on the other side.

Vibration Perception
(Take highest score but both R & L must be abnormal)

0- Vibration felt for >10 seconds (normal)
1- Vibration felt for 6-10 seconds (mild loss)
2- Vibration felt for 5 seconds or less (moderate loss)
3- No feeling of vibration (severe loss)

	Right	Left		
Great toe interphalangeal bone	____	____	(2) use highest value_____/3	nn11

3. INSTRUCTIONS FOR EVALUATING DEEP TENDON REFLEXES:

With the subject seated, the examiner uses one hand to press upward on the ball of the foot, dorsiflexing the subject's ankle to 90 degrees. Using a reflex hammer (long-handled), the examiner strikes the Achilles tendon.

Reflexes
4- Absent
3- Reduced (difficult to elicit)
2- Normal deep tendon reflexes
1- Hyperactive deep tendon reflexes
0- Clonus

	Right	Left		
Ankle Reflexes: (Take highest score but both R & L must be abnormal)	_____	_____	(3) use highest value____/4	nn12

Final score for BPNS (1+2+3) ____/11 nn13

Reduced TNS score: tick in the box :

	0	1	2	3	4	
a. Sensory symptoms from 1a,b,c: Pain, burning pins or numbness	*none*	*Only in toes or soles of feet*	*Symptoms extend to ankle or wrist*	*Symptoms extend to knee or elbow*	*Symptoms knee or elbow or functionally disabling*	nn14
b. Pin sensibility	*normal*	*Reduced in fingers /toes*	*Reduced up to wrist/ ankles*	*Reduced up to elbow/ knee*	*Reduced above elbow/ knee*	nn15
c. Vibration sensibility (use normal as for BPNS)	*normal*	*Reduced in fingers /toes*	*Reduced up to wrist/ ankles*	*Reduced up to elbow/ knee*	*Reduced above elbow/ knee*	nn16
d. Deep tendon jerks	normal	Ankle reflexes reduced	Ankle reflexes absent	Ankle reflexes absent, other reduced	All reflexes absent	nn17
e. Strength- ankle and toes plantar & dorsi-flexion	normal	Mild weakness (MRC 4)	Moderate weakness (MRC 3)	Severe weakness (MRC2)	Paralysis (MRC 0-1)	nn18

Final score for rTNS (a+b+c+d+e) _____/20 nn19

Neuropathy classification according to TNS:

Symptomatic DSP= a ≥1 and b≥1 or c≥1 or d≥1 or significant touch-evoked pain yes/ no _____ n20

Asymptomatic DSP= TNS ≥3 yes/ no _____ nn21

Indeterminate DSP= a ≥1 only yes/ no _____ nn22

Punch Biopsy performed yes/ no _____ nn23

Examination shows another form of peripheral neuropathy (not symmetric distal sensory) yes/no_____ nn24
refer for further evaluation to neuromuscular HIV clinic at GSH (1st wed am of every month; 404-3209)

Proprioception in both toes Normal=0 reduced=1 absent=2 _____ nn25

Appendix 4

Case studies

Four case studies selected from the group of participants with maximal follow-up (20 weeks) are presented. The diversity of presentations and natural histories in these cases highlights the multi-factorial nature of HIV/TB-associated DSP, and the impact of temporal exposures on the clinical picture. The importance of pyridoxine supplementation is highlighted in participants T081 and T096. The impression is that while worsening occurs initially, the overall trend is improvement, particularly with regards to symptoms. An apparent difference in small vs. large fibre natural history may account for this symptomatic improvement despite persistence of neuropathic signs. What is also evident is a dynamic clinical picture that may not have been appreciated if follow-up intervals were more protracted.

Patient T116: rapid-onset asymptomatic ATN

> This 40-year old female patient with a history of two previous episodes of TB, and a history of eight months of ART use two years prior, was seen two weeks after commencing pyridoxine-supplemented (25 mg/day) anti-TB therapy for PTB. Her $CD4^+$ T cell count was 173 and her acetylation status was fast. At baseline, she was experiencing leg cramps, but not classical neuropathic symptoms, and the neuropathy assessment was normal. Two weeks later, she commenced a d4T-based cART regimen and the follow-up assessment was performed after a further two weeks. At this time, she was asymptomatic, but reduced ankle jerks were now demonstrated. Shortly after this, due to the completion of the streptomycin course, d4T was switched to TDF. At subsequent assessments, the ankle reflexes progressed from reduced to absent, following which there was a reduction also in knee deep tendon reflexes; however, no neuropathic symptoms presented, and the rest of the examination was normal. This was paralleled by a gain in weight from 53.8 kg at baseline to 65.9 kg at eight weeks. Immune restitution could not be assessed due to a lack of a follow-up $CD4^+$ T cell count reading. She was discharged home for outpatient completion of the treatment course.

This patient experienced an isolated progressive decrement of deep tendon reflexes soon after commencing d4T. Despite the withdrawal of this agent after just two weeks, the reflex involvement progressed. Being a fast acetylator, her risk for INH-PN was probably low and the temporal relationship to ART favoured a diagnosis of ATN; however, the short duration of exposure

and neuropathic progression despite withdrawal of the agent conflicted with this diagnosis (although this may have been an example of “coasting”. The rapid onset might have been due to “priming” from previous ART exposure and multiple TB episodes with cumulative neurotoxic susceptibility factors.

Patient T081: pyridoxine-responsive INH-PN

> This patient, a 30-year old ART-naïve male with a slow predicted NAT2 phenotype and a $CD4^+$ T cell count of 84, was assessed at baseline nearly two months after commencing regimen 2 anti-TB therapy for TB lymphadenitis. He appeared to have not received pyridoxine until admission five days prior. At baseline, he complained of mild pain and numbness in the toes which he reported had began after starting anti-TB therapy. Examination revealed marked vibration sense impairment and absent ankle jerks plus reduced knee jerks. Four weeks later, after he had commenced an AZT-based cART regimen, he reported improvement in symptoms. Impaired pinprick sensation to the level of the ankles was noted in addition to the previous findings. At the next visit, symptoms had improved again, and he had been prescribed high dose pyridoxine (150 mg/day) in the interim. All reflexes were now absent in addition to the previous findings. In subsequent assessments, the patient continued to report improvement in symptoms, while both vibration and pinprick sensation steadily returned to normal. At the final assessment, the only abnormality was globally absent reflexes.

This patient demonstrated a probable INH-PN. This is supported by his lack of pyridoxine supplementation and improvement once pyridoxine had been initiated, but is not consistent with his intermediate acetylation status, however. A low $CD4^+$ T cell count could possibly have increased his risk for INH-PN. Symptomatic improvement coincided with an initial worsening then improvement of neuropathic signs (other than reflexes); however, improvement was the overall trend, which occurred over a relatively short time period. He remained with a mild asymptomatic large fibre neuropathy.

Patient T048: “TB-DSP” with worsening following anti-TB therapy and non-d-drug ART

> This 30-year old female patient with a $CD4^+$ T cell count of 306 and no history of previous TB or ART use was seen two and a half weeks after commencing therapy for pleural TB. She initially received 25 mg/day of pyridoxine; this was

increased to 100 mg/day on admission. At baseline, her PLP was 134 nmol/l and her 4PA 4380 nmol/l, both well above normal; she was an intermediate acetylator. She was experiencing severe pain, paraesthesia and numbness to the level of the ankles, the onset of which she reported coincided with that of her TB symptoms but before anti-TB therapy, and for which she was receiving amitriptyline 25 mg/day. Examination revealed impaired pinprick sensation to the same level, and reduced ankle jerks. Two weeks after the baseline assessment she commenced a TDF-based cART regimen. At follow-up, her symptoms had worsened, particularly numbness, and had moved proximally, as had the impairment of pinprick sensation. An additional finding at this stage was impaired vibration sense. Her PLP and 4PA were again well over the reference ranges (89.5 and 1540 nmol/l, respectively). At the second follow-up, symptoms and signs had worsened in both severity and anatomical extent. At the third follow-up, symptoms were reported to the level of the knee, signs had also progressed proximally and ankle jerks were absent. Four weeks later; however, the trend was toward improvement: numbness remained as an isolated symptom, and signs moved distally. This trend continued toward the final assessment, at which point she was symptom free, vibration sense was normal, reflexes were elicited (but still reduced) and the primary finding was residual impairment of pinprick sensation to the level of the ankle. Her weight at baseline was 46.5 kg compared to 62.0 kg at discharge.

The reported symptom onset suggested an HIV-DSP (despite a high $CD4^+$ T cell count), or possibly a "TB-DSP", which initially worsened significantly before improving, perhaps another example of "coasting". The worsening was not related to use of d-drug ART, and high dose pyridoxine did not appear to prevent it. A weight gain of 16.5 kg over 5 months might have reflected an immune reconstitution and resulting inflammation contributing to the DSP, particularly in light of the initial worsening followed by improvement, although unlikely because of the high baseline $CD4^+$ T cell count.

Patient T096: worsening INH-PN in an acutely ill patient

T096, a 25-year old male intermediate acetylator with a first episode of PTB, was seen at baseline just over three weeks after commencing pyridoxine-supplemented anti-TB therapy. He was wasted with a BMI of 15.8 and anaemic with an Hb of 7.6, but had no co-morbid conditions other than oral candida. His $CD4^+$ T cell count was 305 and he was ART-naïve. At baseline, he did not complain of any neuropathic symptoms; examination revealed bilateral absent ankle reflexes. Shortly after the baseline assessment, he developed a lung

abscess and pulmonary embolism and was transferred to a secondary hospital. He later returned to DPM where he was seen eight weeks after the baseline assessment. His general condition was poor. He was receiving oral antibiotics and warfarin, and was due to commence TDF-based ART in the following week. He also now complained of mild pain and paraesthesia in the toes, had impaired vibration sensation and demonstrated MRC grade 4 weakness on toe/ankle dorsiflexion, in addition to the absent reflexes noted previously. At the next assessment, his symptoms had resolved, but impaired pinprick sensation to the level of the knees was demonstrated in addition to the previous findings. These signs remained fairly static for the remaining assessments and his clinical condition improved slowly until discharge which followed his completion of six months anti-TB therapy.

Despite a high $CD4^+$ T cell count, the patient had a poor baseline and developed acute complications. This coincided with the development of a symptomatic small fibre neuropathy over a large fibre background. One possible explanation is that his acetylation phenotype was reversed as a result of his acute illness, and that pyridoxine supplementation was not prescribed at the secondary hospital, thereby placing him at high risk for INH-PN. Resolution of his symptoms shortly after readmission to DPM, where pyridoxine supplementation is standard therapy, supports this hypothesis. The findings at baseline are more difficult to account for: the high $CD4^+$ T cell count reduces the likelihood of HIV-DSP (although he had clinical evidence of advanced infection), and no other risk factors for DSP were evident. This highlights the lessons learned from our research in terms of timing of enrolment.

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