Pulmonary Tuberculosis:
towards improved adjunctive therapies

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July 2010

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy of The Australian National University
This thesis describes the development, implementation and preliminary results of the Arginine and Vitamin D Adjunctive Therapy in Pulmonary Tuberculosis (AVDAPT) randomised controlled trial.

I had a central role in developing and implementing the study protocol collaboratively with my PhD supervisors Associate Professor Paul Kelly (Australian National University, Canberra) and Professor Nicholas Anstey (Menzies School of Health Research, Darwin), and with additional input from the investigators listed in Appendix 1. I wrote the initial draft of the study protocol for submission to the relevant ethics committees and for trial registration purposes (http://clinicaltrials.gov/show/ NCT00677339). I supervised and participated in the collection of data, performed the data analyses, wrote the thesis, and wrote all published and submitted manuscripts arising from the thesis. The named co-authors made intellectual and writing contributions to the final manuscripts.

One section of data analysis was not performed by me: the interim safety analysis described in Chapter 10 was conducted by an independent biostatistician (Mr Joseph McDonnell, Menzies School of Health Research) in his role as a member of the AVDAPT study Data and Safety Monitoring Committee.

I am a named Chief Investigator on the successful National Health and Medical Research Council Project Grant Application 605806 entitled “L-Arginine and Vitamin D Adjunctive Therapies in Pulmonary Tuberculosis”.

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July 2010
Acknowledgements

I would like to acknowledge a number of people and organisations who have played significant roles in this research project. I am very grateful to the National Health and Medical Research Council for providing a Postgraduate scholarship and a 2009 research grant, and the Australian Respiratory Council and the Royal Australasian College of Physicians (Covance award) which generously provided funds for the project.

At the field research site in Timika, my very great thanks go to the research assistants Bapak Govert Waramori and Dr Gysje Pontororing, and director of the Timika Translational Research Facility Dr Enny Kenangalem, for their enthusiasm and commitment to the project, and for making the work so enjoyable. Profound thanks also to all Timika Translational Research Facility staff who make the project possible: Ferryanto Chalfein, Prayoga, Daud Rumere, Frans Wabiser, Yeni, Henwi Pieris and Baspak Gobay (laboratory staff); Natalia Dwi Haryanti and Sri Hasunik (data management), Sri Rahayu (administration), and Gertruida Bellatrix and Hendrix Antonius (research assistants). Thanks also to Dr Daniel Lampah for medical and logistical help, and Maikel Zonggonau for driving, errands, and making sense of my limited Bahasa Indonesia. At RSMM, I sincerely thank Dr Paulus Sugiarto for his support and for chairing the Data and Safety Monitoring Committee, and Dr Enny Malonda, for helpful discussions about TB management in Timika. Dr Rini Poespoprodjo and Drs Franciscus Thio have also offered very valuable assistance for which I thank them very much. Special thanks also to TB clinic staff Dr Andri Wiguna for medical support, Bapak Djonny Lempoy for frequent general assistance, and to Bapak Erstanto, Head, Timika TB laboratory, for diligently processing, recording and storing sputum slides for the study. I also greatly thank Drs Pasi Pennitien, Michael Bangs, and Michael Stone (Public Health / Malaria Control, Timika), for their vital support across many tasks, including facilitating access to consumables and transporting specimens to Jakarta. Finally in Timika, I am indebted to all the study participants, healthy volunteers and their families for their involvement in the study.
In Jakarta, I extend my thanks to colleagues at the Ministry of Health’s National Institute of Health Research and Development who allowed this study to proceed. In particular, I thank Dr Sandjaja for his assistance in facilitating the project, and his intellectual and practical input to the project; Dr Dina Bisara Lolong for contributing to the development of the study protocol and visiting the field site, Ibu Merryani Girsang for contributing to laboratory quality control checks, and Dr Emiliana Tjitra for providing a co-ordinating role. Major thanks to Dr Retno Soemanto and Mbak Yuni Rukminiati at the University of Indonesia Faculty of Microbiology for taking on the large task of processing all specimens collected for this project (culture / DST), and for being readily available for frequent discussions about results.

In Darwin at Menzies School of Health Research (MSHR), I wish to convey deep thanks to my supervisor Professor Nicholas Anstey who has been a greatly valued mentor, and whose intellectual rigor is a constant inspiration. His attention to detail and boundless reserves of optimism, tenacity and diplomacy mitigated many potential problems, solved the seemingly insoluble, and kept the project afloat. He also provided greatly-appreciated contributions to the development of this thesis and the publications arising from it. I greatly thank Dr Ric Price (PhD co-supervisor) for trying to impart to me some of his knowledge regarding statistics, data management and data analysis; the databases created for this thesis relied heavily on his assistance, and his detailed statistical advice was greatly appreciated. Great thanks to Kim Piera for her meticulous approach to managing logistics and supplies, providing laboratory expertise, packaging medications, and helping with data entry, as well as providing good company in Timika. Other laboratory personnel including Drs Tonia Woodberry, Gabriella Minigo and Jutta Marfurt have been extremely helpful in educating both myself and the Timika staff in laboratory methods, and striving to maintain good laboratory standards. Administrative staff at MSHR essential to the operation of this project include Tania Paul, Ella Curry, Robi Cohalan, Asriana Kebon and Joanne Bex, to all of whom I am very grateful. Also at MSHR, thanks to Associate Professor Peter Morris (PhD Advisor) for his major contributions to development of the study protocol, advice regarding the analytical plan, and input to devising a composite clinical outcome score; to Dr Nick Douglas for greatly-appreciated help and company in Timika; to Dr Tsin Yeo for statistical advice and for making available his data and knowledge on exhaled nitric oxide measurement; to Dr
Joshua Davis for friendly and comprehensible statistical advice; to Dr Louise Maple-Brown and Joseph McDonnell for their roles in the Data and Safety Monitoring Committee.

At ANU, immense thanks to Associate Professor Paul Kelly who chaired my PhD supervisory panel. It was through enthusiastic discussions with him in 2006 that I was inspired to embark on this research. His earlier tuberculosis work in Timika and in other international settings provided a strong basis for the current project, and his contributions to discussions with collaborators in Timika and Jakarta have been vital for the project’s operation. I also sincerely thank Paul for making available to me the TB data that he and others had previously obtained in Timika, for providing templates on which I could model the study protocol and data collection forms, and for providing essential feedback on manuscripts, including this thesis. My thanks also go to Professor Niels Becker (PhD co-supervisor) for providing patient and detailed statistical help, especially in relation to the x-ray analyses and the exhaled nitric oxide correction factors, and to Dr Mark Clemens for his major assistance in the complicated factorial study sample size calculation. To all other ANU staff who provided help (including Dr Robyn Lucas for vitamin D advice, and Sarah Geddes and Barbara Bowen for administrative help), to many fellow ANU PhD students who provided much-appreciated camaraderie and support, I convey my great thanks also.

I also wish to sincerely thank Associate Professor Graeme Maguire, James Cook University (PhD Advisor), for support with supplies, logistics, lung function testing, data analyses, and occasional accommodation (snakes notwithstanding). I am very grateful for the important mycobacteriological advice and expertise provided by Mr Richard Lumb at the Institute for Medical & Veterinary Science, including his visits to the Jakarta laboratory. Many thanks also to Dr Cheryl Salome, Woolcock Institute of Medical Research, for valuable exhaled nitric oxide advice and kind provision of materials. Thanks also to Dr Mairwen Jones for proof reading sections of this thesis.

Deepest thanks finally go to my partner Deborah for tolerating my absences and other difficulties this project has brought, and for supporting me unconditionally in every way.
FIGURE 1: NIHRD-MSHR Timika research staff outside the research building

L to R: Front row Maikel Zonggonau, Enny Kenangalem, Sri Rahaya, Basbak Gobay, Hendrix Antonius
The potential to improve pulmonary tuberculosis (PTB) treatment outcomes with adjunctive immunotherapies requires investigation. L-arginine and vitamin D have antimycobacterial properties which render them suitable candidates. Therefore the Arginine and Vitamin D Adjunctive therapy in Pulmonary TB (AVDAPT) trial evaluates these supplements in PTB. This large trial commenced in June 2008. The project is run in Timika, Papua Province, Indonesia by the International Health Division, Menzies School of Health Research (Darwin, Australia), the National Institute for Health Research and Development (Ministry of Health, Indonesia), and the Australian National University (Canberra).

Aims of this thesis were to design and commence the AVDAPT study and examine baseline data. Among the tested hypotheses were that exhaled nitric oxide (FENO), an L-arginine-derived antimycobacterial immunological mediator, would be elevated in PTB compared with healthy controls (HC), and inversely related to disease severity; secondly, that significant relationships would exist between different measures of TB severity.

Consenting, eligible adults with smear-positive PTB were enrolled at the Timika TB clinic according to the protocol. Assessments included sputum microscopy, culture and susceptibility, X-ray, weight, pulmonary function, FENO, 6-minute walk testing (6MWT) and quality of life (St George’s Respiratory Questionnaire [SGRQ]). HC were enrolled for a single assessment.

Results from 162 TB patients and 40 HC included: (1) findings pertaining to the trial (development / validation of outcome measures, and establishment of locally-relevant reference ranges for 6MWT and SGRQ); (2) findings pertaining to improved understanding of TB (demonstration of relationships between clinical, physiological, immunological [FENO] and functional measures of disease severity), and (3) investigation of TB drug-resistance and HIV rates.
A key finding was that FE\textsubscript{NO} was not elevated in TB compared with HC and was lower still in worse disease. These findings suggest that an impaired ability to generate adequate NO (e.g. in L-arginine deficiency) might contribute to host inability to adequately contain TB or mitigate lung pathology. These findings support the rationale for conducting a trial of adjunctive L-arginine in TB.

New relationships were identified between sputum smear grade, X-ray, weight, pulmonary function, 6MWT and SGRQ. Patients with more-severe malnutrition had worse pulmonary function; 6MWT was independent of lung function; SGRQ results accurately captured people’s perceived quality of life, correlating significantly with symptoms, 6MWT and pulmonary function; and sputum bacillary grade was significantly related to radiological extent and weight, but not to other results. These findings support the use of a range of outcome measures in TB trials, to provide a comprehensive assessment of TB severity, rather than focusing on bacteriology alone.

An x-ray severity score and a clinical outcome score were created, providing valuable tools for use in clinical trials. Interim analysis confirmed the safety of L-arginine and vitamin D adjunctive therapy. Multi-drug resistant TB rates remained low in new cases (2.0%), but HIV-TB co-infection rates rose significantly over 5 years, creating major challenges.

This thesis provides the basis for continuation of the AVDAPT study, produces original findings relating to clinico-immunological aspects of PTB, and provides information of major local importance to help guide TB service provision in Timika.
Glossary

6MWT  6 minute walk test
6MWWD  6 minute weight.walk distance
AE  Adverse event
AVDAPT  Arginine and Vitamin D Adjunctive therapy in pulmonary tuberculosis
AFB  Acid-fast bacilli
AIDS  Acquired immunodeficiency syndrome
ANU  Australian National University
BTA  Basil tahan asam (acid-fast bacilli)
CI  Confidence interval
Dinas  Dinas Kesehatan (District Health Authority)
DOT  Directly Observed Treatment
DOTS  Directly Observed Treatment, Short-course
DSMC  Data and Safety Monitoring Committee
E  Ethambutol
FDC  Fixed-dose combination antituberculous therapy
$\text{FE}_{\text{NO}}$  Fractional exhaled nitric oxide
FEV$_1$  Forced expiratory volume in 1 minute
FKUI  Faculty of Microbiology, University of Indonesia
GMP  Good Manufacturing Practice
H  Isoniazid
HIV  Human Immunodeficiency Virus
IFN-γ  Interferon gamma
IMVS  Institute for Medical & Veterinary Science
MDR-TB  Multi-drug resistant TB
MGIT  Mycobacterium Growth Indicator Tube
MIRU  Micro-satellite Interstitial Repetitive Unit
MSHR  Menzies School of Health Research (Darwin, Australia)
MTB  Mycobacterium tuberculosis
NCEPH  National Centre for Epidemiology & Population Health (Australia)
NHMRC  National Health & Medical Research Council (Australia)
NIHRD  National Institute of Health Research & Development (Indonesia)
NiOX FLEX  Device for measurement of exhaled nitric oxide (non-portable)
NiOX MINO  Device for measurement of exhaled nitric oxide (portable)
NO  Nitric oxide
NOS  Nitric Oxide synthase
NTP  National TB Control Program
PTB  Pulmonary TB
PT  Perseroan Terbatus (Proprietary Limited)
Puskesmas  Pusat Kesehatan Masyarakat (Community Health Centre)
R  Rifampicin
RA  Research assistant
RCT  Randomised, controlled trial
RSMM  Rumah Sakit Mitra Mayarakat (Community hospital, Timika)
RSUD  Rumah Sakit Umum Daerah (Regional General Hospital, Timika)
S  Streptomycin
SAE  Serious adverse events
SGRQ  St George’s Respiratory Questionnaire
TB  Tuberculosis
Th1  T helper cell Type 1
TLR  Toll-like receptor
TNF  Tumour necrosis factor
UV  Ultraviolet
VCT  Voluntary counselling and testing for HIV
WHO  World Health Organization
XDR-TB  Extensively drug-resistant TB
Z  Pyrazinamide
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PUBLISHED AND SUBMITTED MANUSCRIPTS

Manuscript 1: Ralph AP, Anstey NM, Kelly PM. Tuberculosis into the 2010s: is the glass half full? Clin Infect Dis 2009;49(4):574-83 .......................................................... 3
1 Aims

1.1 BACKGROUND

*Mycobacterium tuberculosis* (MTB) is one of the most successful human pathogens. It infects an estimated third of the human population, and currently accounts for the greatest number of deaths from a curable infectious disease.\(^1\)\(^,\)\(^2\) Tuberculosis (TB) is an historical disease whose existence may pre-date *Homo sapiens*,\(^3\) but it maintains major contemporary relevance. It re-emerged as a ‘global emergency’ by the turn of the 21\(^{st}\) century, and now is defying reduction targets in parts of the globe worst-affected by the overlapping pandemics of TB and HIV.\(^2\) New case numbers are estimated at around 9.3 million annually.\(^2\) Antibiotic treatment regimens for TB, largely unchanged in the last four decades, are cumbersome and protracted, contributing to cure rates frequently falling short of the 85% target set by the World Health Organisation (WHO).\(^4\)

Innovative strategies therefore require investigation for their potential to accelerate responses to antibiotics, with the ultimate goals of reducing required TB treatment duration, reducing the period of infectivity, permitting earlier return to employment or school, and reducing post-TB residual lung pathology.

Recognising this priority research field, the AVDAPT (Arginine and Vitamin D Adjunctive therapy in Pulmonary TB) clinical trial investigates the use of L-arginine and vitamin D as immunotherapies supplementary to conventional TB treatment in pulmonary TB. This is a large randomised, double-blind, placebo-controlled trial which commenced in June 2008 and is projected to complete enrolments in early 2012, at the Timika Translational Research Facility in Papua, Indonesia, through Menzies School of Health Research’s (MSHR) International Health Division in partnership with the National Institute for Health Research and Development (NIHRD), Indonesian Ministry of Health, and the Australian National University (ANU).
1.2 AIMS

The aim of this thesis is to design and implement a clinical trial of the safety and efficacy of L-arginine and vitamin D as adjunctive therapies in pulmonary TB (the AVDAPT study). This includes methodological objectives (Aim 1) and analytical objectives (Aims 2 to 8).

In detail, this comprises development of methodologies relevant to operating the trial, testing of hypotheses regarding the validity of the measures used in the study, examination of data collected during the initial phase of study participant recruitment and follow up (June 2008 - October 2009), and determination of longitudinal epidemiological trends by comparing current data with an historical cohort at the same site.

Final results of the AVDAPT study will be presented at completion of the trial, anticipated to be in 2012.

AIM 1: DESIGN AND COMMENCE THE AVDAPT RCT

The first objective is to design and commence the AVDAPT RCT. This study aims to determine whether supplementation with L-arginine and / or vitamin D is safe and effective in TB, where efficacy includes more rapid improvement in clinical, mycobacterial, immunological, radiological, physiological and / or functional measures of treatment outcome. In order to gain understanding of the underlying immunology of relevance to this trial, the AVDAPT study further aims to determine whether exhaled nitric oxide (FE\textsubscript{NO}) is inversely related to disease severity. The detailed aims of the AVDAPT trial are set forth in Chapter 6 (Methods). The hypotheses investigated in this study include:

*Hypothesis 1(a):* That L-arginine supplementation in pulmonary TB will be safe, will increase plasma arginine concentrations, will enhance pulmonary production of nitric oxide (NO) (a key arginine-dependent immunomodulator and downstream immune mediator of mycobacterial killing) and will improve the rapidity and magnitude of the microbiological and clinical response. Baseline pulmonary NO production will be elevated in pulmonary TB but inversely associated with disease severity. Both
baseline and post-treatment increments in exhaled NO will be associated with rapidity and magnitude of the treatment response.

*Hypothesis 1(b):* That supplementation with vitamin D, the metabolite of which (1,25-dihydroxyvitamin D₃) has anti-mycobacterial activity, will be safe, will increase plasma vitamin D concentrations, and will improve the rapidity and magnitude of the treatment response in human PTB.

**AIM 2: INVESTIGATE BASELINE CHARACTERISTICS OF TB STUDY PARTICIPANTS AND HEALTHY VOLUNTEERS**

*Aim 2(a):* to explore the baseline demographic characteristics of AVDAPT study participants, and present clinical and laboratory findings at the time of their enrolment into the study, including results of *Mycobacterium tuberculosis* susceptibility testing.

*Aim 2(b):* to establish reference ranges for exercise tolerance (six-minute walk test) and a quality of life score (modified SGRQ), by performing these tests in locally recruited healthy volunteers, for comparison with values obtained in TB patients (AVDAPT study participants).

**AIM 3: INVESTIGATE RELATIONSHIPS BETWEEN MICROBIOLOGICAL, CLINICAL AND FUNCTIONAL MEASURES AT BASELINE**

*Aim:* to describe the relationships between clinical (symptoms and weight), mycobacterial (sputum smear grade), physiological (spirometry) and functional (six-minute walk test, SGRQ) measures, and to investigate any associations between socio-economic indicators and diagnostic delay or disease severity.

*Hypotheses:* That clinical and functional measures of TB disease severity at enrolment will significantly relate to bacteriological (sputum smear grade) measures. Specifically, that baseline weight, FEV₁ and six-minute walk test will be *inversely* related, and modified SGRQ score, cough severity and number of symptoms will be *directly* related, to sputum smear grade. Also, that clinical, functional and physiological measures of TB disease severity at enrolment will significantly correlate with each other.
AIM 4: MEASURE RADIOLOGICAL SEVERITY OF TB

Aim: To develop a valid method by which to grade chest X-ray severity in study participants with pulmonary TB, using a previously-collected dataset from a similar sample of adults with pulmonary TB in Timika, and to further examine the ability of this score to predict baseline clinical and microbiological severity and 2 month outcomes in AVDAPT study set.

Hypothesis: that a numerical score applied to an X-ray can provide a means of evaluating baseline severity and response to treatment.

AIM 5: DEVELOP A COMPOSITE CLINICAL OUTCOME MEASURE

Aim: to develop a composite clinical outcome score calculable at 2 and 6 months, and determine the relationship between this and microbiological, radiological and functional measures of TB severity

Hypothesis: That a composite clinical outcome score at 2 and 6 months will be significantly related to microbiological, radiological and functional measures.

AIM 6: EVALUATE INTERIM OUTCOMES

Aim: to document treatment outcomes among study participants, including sputum smear and culture conversion, and provide interim adverse event and safety data relating to the AVDAPT study.

AIM 7: MEASURE EXHALED NITRIC OXIDE IN PULMONARY TB

Aims: to determine the relationship between FENO and TB disease severity (bacteriological, radiological and clinical) at TB diagnosis; to compare FENO measures from pulmonary TB patients with measures from contemporaneously evaluated local healthy controls and an historical healthy control group from the same research site; and to determine longitudinal changes in FENO in response to TB treatment.
Hypothesis: That $\text{Fe}_{\text{NO}}$ will be increased in participants with pulmonary TB compared with healthy controls, will be inversely related to disease severity at baseline, and will return towards normal by the end of therapy.

AIM 8: EPIDEMIOLOGY OF HIV–TB COINFECTION

Aim: to describe the current epidemiology of HIV-TB co-infection in Timika, and compare the HIV-TB co-infection rates in 2008-2009 with 2003-2004 (a time period for which previous HIV-TB co-infection rates are published).
TB has been the subject of recent comprehensive reviews.\textsuperscript{5,7} My approach to reviewing the literature in this introductory chapter is therefore to summarise milestones in TB understanding and management (Table 2.1), and to focus on two specific questions: 1) What recent progress has been made globally in TB control? 2) What should Australian practitioners know about contemporary TB management? These questions are addressed, respectively, in publications reproduced hereafter: “TB into the 2010s: is the glass half full?”\textsuperscript{8} and “What’s new in TB?”\textsuperscript{9}

Table 2.1: Chronology of TB milestones

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1882</td>
<td>Robert Koch (German physician), announced his identification of \textit{Mycobacterium tuberculosis} as the causative agent of TB on March 24\textsuperscript{th}, now commemorated as World TB Day.</td>
</tr>
<tr>
<td>1882</td>
<td>German pathologists Ziehl and Neelsen introduced a staining method using carbolfuchsin, an acid wash, and methylene blue to demonstrate the presence of acid-fast bacilli.</td>
</tr>
<tr>
<td>1890</td>
<td>Description of tuberculin by Koch, trialed (unsuccessfully) to treat TB.</td>
</tr>
<tr>
<td>1895</td>
<td>Discovery of x-ray techniques for diagnostic purposes by Conrad Roentgen, providing an important additional diagnostic tool for pulmonary TB.</td>
</tr>
<tr>
<td>1905</td>
<td>Koch received Nobel Prize in Physiology or Medicine.</td>
</tr>
<tr>
<td>1921</td>
<td>Development of vaccine, now known as BCG, from attenuated \textit{Mycobacterium bovis} by Calmette and Guerin.</td>
</tr>
<tr>
<td>1940s</td>
<td>Roll-out of mass TB vaccination using BCG.</td>
</tr>
<tr>
<td>1944</td>
<td>Streptomycin (SM) and Para-amino salicylic acid (PAS) discovered as effective antimicrobial therapies for the treatment of TB.</td>
</tr>
<tr>
<td>1947-</td>
<td>First randomised curative trial to be conducted in the UK was performed, evaluating SM as an anti-tuberculosis agent.</td>
</tr>
<tr>
<td>1948</td>
<td>Addition of isoniazid (INH) to SM and PAS shown to increase cure rates from 70 to 95%, but required treatment for 18-24 months.</td>
</tr>
<tr>
<td>1952</td>
<td>Madras study showed domiciliary treatment to be as good as in sanitaria, and not resulting in more TB cases in family contacts, allowing TB treatment to be shifted to the community.</td>
</tr>
<tr>
<td>1965</td>
<td>Rifampicin discovered, leading to the advent of short course combination therapy.</td>
</tr>
<tr>
<td>Year</td>
<td>Event</td>
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<tr>
<td>------</td>
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</tr>
<tr>
<td>1969</td>
<td>Given successes in the management of TB and other infectious diseases, U.S Surgeon General William Stewart famously stated in his address to Congress it was time to “close the book on infectious diseases”. This prevailing attitude led to TB-specific control being dismantled in many countries during the 1970-80s.</td>
</tr>
<tr>
<td>1981</td>
<td>HIV pandemic first recognized.</td>
</tr>
<tr>
<td>1985</td>
<td>TB incidence in the USA began to rise for first time in 30 years, described as an unprecedented resurgence during 1985-1992.</td>
</tr>
<tr>
<td>1991</td>
<td>World Health Organisation (WHO) specifies targets for case detection and cure: detect at least 70% and cure at least 85% of smear-positive TB cases.</td>
</tr>
<tr>
<td>1991</td>
<td>WHO Directly Observed Therapy, Short Course (DOTS) strategy rolled out (officially named ‘DOTS’ in 1994) comprising: (1) Political commitment with increased, sustained financing; (2) Case detection through quality-assured bacteriology; (3) Standardized treatment, with supervision and patient support; (4) An effective drug supply and management system; (5) Monitoring and evaluation system, and impact measurement.</td>
</tr>
<tr>
<td>1992</td>
<td>Multi-drug resistant (MDR)-TB identified as a major new threat in New York City.</td>
</tr>
<tr>
<td>1993</td>
<td>World Health Assembly declared TB a “Global Emergency”.</td>
</tr>
<tr>
<td>2003</td>
<td>Early guidelines released on the use of interferon gamma release assays for latent TB diagnosis; understanding of test interpretation evolved during the remainder of the decade.</td>
</tr>
<tr>
<td>2006</td>
<td>Extensively drug-resistant TB labeled ‘XDR-TB’. XDR-TB / HIV co-infection in Kwa-Zulu Natal province, South Africa, reported to have near 100% fatality.</td>
</tr>
<tr>
<td>2006</td>
<td>Stop TB partnership launched.</td>
</tr>
<tr>
<td>2008</td>
<td>WHO endorsed the use of rapid molecular resistance detection tests, able to provide MDR-TB diagnosis in 1-2 days.</td>
</tr>
<tr>
<td>2008</td>
<td>MDR and XDR-TB cure rates of &gt;60% reported.</td>
</tr>
<tr>
<td>2008</td>
<td>Mortality significantly reduced in HIV-TB co-infection by commencing antiretroviral therapy during TB treatment instead of deferring until after TB treatment.</td>
</tr>
<tr>
<td>2009</td>
<td>Positive results from drug trials (e.g. moxifloxacin, TMC207) provide optimism for improvements of standard TB regimens and MDR-TB treatment.</td>
</tr>
</tbody>
</table>

Table 2.1 references 5,10-20
Manuscript 1: Ralph AP, Anstey NM, Kelly PM. Tuberculosis into the 2010s: is the glass half full? Clin Infect Dis 2009;49(4):574-83.

<see paper next page>
Tuberculosis into the 2010s: Is the Glass Half Full?

Anna P. Ralph,1,4 Nicholas M. Anstey,2,3 and Paul M. Kelly1,4

1International Health Division, Menzies School of Health Research, 2Charles Darwin University, and 3Division of Medicine, Royal Darwin Hospital, Darwin, and 4National Centre for Epidemiology and Population Health Research, College of Medicine, Biology and Environment, Australian National University, Canberra, Australia

During the 16 years since the World Health Organization declared tuberculosis (TB) a global emergency, major new challenges have emerged—in particular the spread of extensively drug-resistant (XDR)-TB and its overlap with human immunodeficiency virus infection. However, during this period, we have also witnessed the creation of—and major commitments from—agencies dedicated to TB control, research, and funding, and tangible positive achievements have occurred; these include improvements in both new and existing TB diagnostics, a developmental pipeline of new candidate TB drugs, better treatment outcomes for multidrug-resistant TB and XDR-TB, heightened recognition of the importance of nosocomial transmission, and improved strategies to reduce mortality associated with concurrent human immunodeficiency virus infection and TB. We suggest updates to the 2006 International Standards of Tuberculosis Care to embrace these developments. The incorporation of these recent advances into optimized directly observed treatment, short course (DOTS), programs, in conjunction with more widespread deployment and enhanced political will, all provide grounds for improved control.

Mycobacterium tuberculosis is the consummate human pathogen. Millennia of evolution alongside human hosts have led to elaborate immune evasion and transmission strategies [1, 2]; as such, M. tuberculosis is thought to infect one-third of humans, and in 2007, it accounted for an estimated 9.27 million new cases of tuberculosis (TB) and ∼1.7 million deaths [3]. Compounding this already crippling burden are the expanding threats of TB drug resistance and of concurrent human immunodeficiency virus (HIV) infection and TB. Primary transmission is the most common mode of acquisition seen in some settings for both extensively drug-resistant (XDR) and multidrug-resistant (MDR) TB [4–6]. The overlapping of HIV and TB epidemics in sub-Saharan Africa in particular creates a health care crisis and renders it unlikely that the Millennium Development Goal 6.C of reduction in TB prevalence and mortality by 50% by 2015 will be achievable in this region [7, 8].

Amid these grim realities, however, exciting recent developments with the potential to bring about important reductions in TB-related burden of disease have been achieved, including in underresourced settings. However, their implementation poses new challenges due to resource constraints, policy-change inertia, and the need to prioritize basic TB care, as articulated by the World Health Organization’s (WHO’s) directly observed therapy, short-course (DOTS), and Stop TB partnership strategies [9, 10]. As has been clearly articulated, it is incumbent upon those with expertise and resources to take a serious role in bringing these developments into action in underresourced TB-burdened settings [11]. Indeed, investing in TB control in resource-poor settings might be more cost-effective for developed nations in improving their own TB control than alternative approaches [12].

The TB literature is characterized by bleak statistics that provide substance for, in health-promotion terms, "fear-based" appeals; there is merit in directing attention in this manner to the dire global state of TB. However, there is an equal place for the alternative approach...
of positive appeals as effective strategies to promote a shift in mindset and uptake of new practices [13]. Here, we review important recent gains made in TB management and knowledge, discuss how these might be incorporated into existing DOTS programs, suggest a revision of several standards contained within the comprehensive 17-point 2006 International Standards of TB Care (ISTC), and recommend an 18th Standard [14]. Contrasting with negative appeals, we show that there is scope for optimism.

**TB DIAGNOSTICS**

Rapid recent developments have occurred in the field of TB diagnostics, as evidenced by the need to establish a subgroup within the Stop TB Partnership’s New Diagnostics Working Group to provide ongoing systematic reviews of diagnostic methods (Table 1) [15].

Of greatest priority are affordable ways to improve case detection through smear microscopy at field laboratories. Simple procedures recently shown to be of benefit include more clear instruction of people on how to produce an adequate sputum specimen [16], a reduction in the required number of specimens from 3 to 2 [17], more rapid specimen collection [18], and the processing of sputum specimens prior to examination (Table 1) [15, 19]. Fluorescence microscopy is more sensitive and rapid than conventional microscopy [19], and a light-emitting diode light source has recently been shown to be a reliable alternative to the expensive, short-lived mercury vapor lamps [20]. Such approaches provide solutions to the valid assertion that substandard TB diagnostics are unacceptable in resource-poor countries [11].

Transportation of specimens to a reference laboratory can be achieved despite challenging barriers: fresh sputum samples can be stored at 4°C for up to 6 weeks before unrefrigerated transportation, achieving excellent M. tuberculosis recovery without excessive contamination [21]. At laboratories with adequate capacity, early resistance detection—a critical tool in prevention of resistance amplification that is associated with better treatment outcomes for management of MDR-TB [22]—is now achievable with rapid molecular and culture-based methods. Line-probe assays (eg, Genotype MTBDRplus assay [Hain Lifescience]), which detect M. tuberculosis gene mutations that confer resistance to rifampicin (rpoB) and isoniazid (katG and inhA), provide sensitive and specific results in 6 h to 2 days [23–25]. Culture-based rapid methods for resistance detection are outlined in Table 1.

These improvements over traditional direct Ziehl-Neelsen staining and use of solid-culture media require rapid dissemination and uptake. Promotion of new technologies, via WHO endorsement [25], inclusion in guidelines [26], or internationally accepted standards [14], is the first step in their deployment, but innovative means are required to traverse the formidable barriers to dissemination of these messages to national TB control programs and the practitioners who implement these programs.

**NEW DRUG REGIMENS**

New anti-TB drugs are required to permit shorter treatment durations for drug-susceptible TB [27], which mathematical modeling indicates could effect major reductions in TB incidence and mortality [28], and to provide less toxic, more effective, and shorter regimens for MDR-TB. The TB literature has long lamented the absence of new drug developments since rifampicin in the 1960s. Barriers to TB drug development include the need to evaluate drugs in combination over long follow-up periods in resource-limited settings; a lack of good animal models for preclinical drug evaluation [29]; metabolic adaptability of M. tuberculosis, whereby genetic targets that appear promising have not proven to be so [30]; and the perception by pharmaceutical companies that antibiotic development is unrewarding [31]. Modeling performed in 2006 that incorporated the high attrition in drug development, found only a 5% chance of a new TB drug being ready for clinical use in humans by 2010 [32]. Despite these impediments, there are now >30 new drugs for TB under development (Table 2) [29, 33–35]. PA-824 (a nitroimidazole) was shown to be successful against M. tuberculosis in vitro and in mouse models [36] and is now in phase II human trial stage (http://www.clinicaltrials.gov/show/NCT00567840). The diarylquinoline TMC207 (also called R207910) has appeared to be particularly promising in animal studies [37, 38], and a phase IIa randomized trial involving people with smear-positive pulmonary TB has been recently completed (http://www.clinicaltrials.gov/show/NCT00523926). Benzothiazinones (eg, BTZ043) are a propitious new antimycobacterial class which kill M. tuberculosis in vitro and in mice by targeting M. tuberculosis cell wall synthesis [39], and have important potential in drug-susceptible and drug-resistant TB.

The requirement for prolonged treatment has been hypothesized to arise from the development of a nonreplicating, phenotypically drug-resistant phase of M. tuberculosis driven by hypoxia and low-level nitric oxide; these factors, which characterize the internal environment of granulomata, up-regulate dormancy genes to yield the metabolically inert state [30, 40–42]. To shorten treatment regimens, new drugs inhibiting mechanisms underlying this nonreplicating state may have promise [30].

A second strategy that may permit shorter TB treatment regimens is the use of drugs that are bacteriocidal against rapidly metabolizing M. tuberculosis. Replacement of etambutol with moxifloxacin has been shown to significantly increase the 2-month sputum culture conversion rate, from 63% to 80% [43]. Both moxifloxacin and gatifloxacin improved the sterilizing
Table 1. Methods to Improve Diagnosis and Accelerate Drug Susceptibility Results

<table>
<thead>
<tr>
<th>Method, test</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sputum collection</strong></td>
<td></td>
</tr>
<tr>
<td>Improved sputum-submission guidance</td>
<td>If smear positive pulmonary TB case detection is impaired by poor-quality specimen submission, case detection can be improved by provision of adequate instructions</td>
</tr>
<tr>
<td>Reduce number of collections from 3 to 2</td>
<td>Because incremental yields from subsequent sputum specimens are small, WHO recommends examining 2 smears; this can alleviate laboratory workloads, decrease time for diagnosis, and decrease the number of patients who &quot;drop out&quot; of the diagnostic pathway</td>
</tr>
<tr>
<td><strong>Sputum smear microscopy</strong></td>
<td></td>
</tr>
<tr>
<td>Processing of sputum sample prior to smear examination (eg, use of bleach then centrifugation or use of bleach or sodium hydroxide then overnight sedimentation)</td>
<td>This is 18%–23% more sensitive than direct microscopy</td>
</tr>
<tr>
<td>Fluorescence microscopy</td>
<td>This is 10% more sensitive than conventional microscopy; use to determine viability of Mycobacterium tuberculosis in follow-up sputum specimens to detect treatment failure</td>
</tr>
<tr>
<td>Fluorescence microscopy using light-emitting diode light source</td>
<td>These light sources are cheaper, last longer, and have less potential for environmental contamination than do traditional lamps used in this method</td>
</tr>
<tr>
<td><strong>Culture-based methods</strong></td>
<td></td>
</tr>
<tr>
<td>Liquid culture (eg, automated mycobacteria growth indicator tube)</td>
<td>Faster and more sensitive than solid media; recommended standard practice</td>
</tr>
<tr>
<td>Microscopic observation drug susceptibility assay</td>
<td>Yields faster culture and DST results than do liquid or solid media and is inexpensive, but requires inverted microscope and skilled technician to interpret culture appearance of M. tuberculosis</td>
</tr>
<tr>
<td>Thin-layer agar methodology</td>
<td>Yields faster culture and DST results than do liquid or solid media and is inexpensive, but requires skilled technician to recognize M. tuberculosis colony formation.</td>
</tr>
<tr>
<td>Colorimetric DST methods using redox indicators, tetrazolium salts, or a nitrate reductase assay</td>
<td>These are low-cost, low-tech, and able to yield DST results within 2 weeks; potential for biosafety hazard</td>
</tr>
<tr>
<td><strong>Molecular methods</strong></td>
<td></td>
</tr>
<tr>
<td>Line probe assays (eg, Genotype MTBDRplus assay [Hain] and INNO-LiPA Rif.TB assay [Immunogenetics])</td>
<td>High sensitivity and specificity for detection of rifampicin (with or without isoniazid) resistance, with a 1–2 day turnaround time directly for smear-positive sputum; requires DNA extraction and amplification facilities</td>
</tr>
<tr>
<td>Nucleic acid amplification tests</td>
<td>High specificity; important role in confirming mycobacterial identity; poor negative predictive value for pulmonary and extrapulmonary TB; updated US CDC guidelines recommend sputum M. tuberculosis nucleic acid amplification tests for cases of suspected, unconfirmed TB if results would alter management [111]</td>
</tr>
<tr>
<td>Cytokine assays: T cell interferon-γ release assays</td>
<td>Useful in targeted strategies for LTBI detection in low TB-incidence settings; more specific than tuberculin skin test; cannot distinguish active from treated TB or LTBI</td>
</tr>
</tbody>
</table>

**NOTE.** From [15–17, 19, 20, 112, 113]. CDC, Centers for Disease Control and Prevention; DST, drug susceptibility testing; LTBI, latent tuberculosis infection; TB, tuberculosis; WHO, World Health Organization.

activity of regimens for drug-susceptible TB [27] (gatifloxacin has since been withdrawn from some markets on the basis of adverse glycemic effects). Although, in a multicenter study, moxifloxacin failed to accelerate sputum culture negativity at 2 months, 1-month culture conversions were more frequently achieved [44]. The later-generation fluoroquinolones have a well-established place in the treatment of MDR-TB; the possibility that they might also permit shorter treatment durations for drug-susceptible TB is lent credibility by these recent findings.

**VACCINES AND ADJUNCTIVE IMMUNOTHERAPIES**

The need for an improvement on bacille Calmette-Guérin has been long recognized as a major priority [45]. The status of
Table 2. Antituberculosis Agents in Current Use and in Development

<table>
<thead>
<tr>
<th>Current antituberculosis agents, WHO classification [26]</th>
<th>Agent</th>
<th>Agents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 First-line oral antituberculosis agents</td>
<td>Isoniazid, rifampicin, ethambutol, pyrazinamide, rifabutin</td>
<td></td>
</tr>
<tr>
<td>Group 2 Injectable antituberculosis agents</td>
<td>Streptomycin, kanamycin, amikacin, capreomycin</td>
<td></td>
</tr>
<tr>
<td>Group 3 Fluoroquinolones</td>
<td>Moxifloxacin, levofloxacin, ofloxacin</td>
<td></td>
</tr>
<tr>
<td>Group 4 Second-line oral bacteriostatic antituberculosis agents</td>
<td>Ethionamide or prothionamide, cycloserine or terizidone, para-aminosalicylic acid</td>
<td></td>
</tr>
<tr>
<td>Group 5 Antituberculosis agents with unclear efficacy</td>
<td>Clofazimine, amoxicillin-clavulanate, linezolid, thioacetamide (contraindicated for HIV-infected patients), imipenem-cilastin, high-dose isoniazid, clarithromycin</td>
<td></td>
</tr>
<tr>
<td>New drugs</td>
<td>Diarylquinolone (TMC-207, also known as R207910), nitrimidazopyrans (PA-824 and OPC-67883), diamine (SQ-109)</td>
<td></td>
</tr>
<tr>
<td>Group 6 New drugs undergoing clinical evaluation</td>
<td>Benzothiazinones (eg, BTZ043), LL-3858, cell-wall inhibitors, multifunctional molecules, diaryl oxides, dihydrolipoamide acyltransferase inhibitors, dipiperidine SQ-609, econazole, fatty acid synthase inhibitors, hydrazones, InhA inhibitors, isocitrate lyase inhibitors, malate synthase inhibitors, mefloquine analogues, oxazolidinones, peptide deformylase inhibitors, plumeromutins, riminophenazines, thioacetamide inhibitors, topoisomerase inhibitors</td>
<td></td>
</tr>
<tr>
<td>Group 7 New drugs at discovery stage</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

NOTE. Multidrug-resistant tuberculosis treatment regimens should use all first-line drugs to which the organism is susceptible, a fluoroquinolone, a daily injectible (for at least 4 months after culture conversion and for at least 6 months in total), and other second-line drugs to provide at least 4 agents (for minimum of 18 months), with directly observed therapy, short course (DOTS), used throughout [26, 76]. In drug susceptibility testing-tailored regimens, mindfulness of the lack of universal clinical applicability of all drug susceptibility test results and cross-resistance is required [26]. From [26, 29, 33, 35, 38, 39]. HIV, human immunodeficiency virus.

promising candidate recombinant TB vaccines, including the commencement of phase II trials of MVA85A, has been reviewed in detail elsewhere [45–47].

Investigation of adjunctive immunotherapies is identified as one of several priority research areas [48–51]. They offer an attractively novel strategy to shorten TB treatment and to conserve antimicrobial efficacy in the face of growing resistance. Despite negative results from many studies using inactivated Mycobacterium vaccae as an adjunct to TB chemotherapy [52, 53], hope persists that this immunotherapy given in multiple doses might yet have potential as an adjunctive treatment in MDR-TB or previously treated TB [54] or as a preventative vaccine in previously bacille Calmette-Guérin–vaccinated HIV-infected persons [55, 56].

The concept of micronutrients as potential adjunctive immunotherapeutic candidates has gained currency since specific antimycobacterial mechanisms of action of vitamin D₃ in macrophages (chiefly, up-regulation of LL-37/cathelicin) were demonstrated [57, 58]. The amino acid L-arginine has been found to influence antimycobacterial T cell responses via expression of the zeta chain of the CD3/T-cell receptor complex [59], and the metabolic product of L-arginine, nitric oxide, mediates important macrophage antimycobacterial responses [58]. Some multiple-micronutrient interventions in TB have been associated with benefits in patient subgroups or in non-bacteriological end points [60–62]. Whole-food nutritional support poses logistical challenges and has yet to be shown to be cost-effective or to improve bacteriological outcomes, but it can improve weight and possibly other parameters [63, 64]. The WHO recommends nutritional support in the management of MDR TB [26], and many programs already incorporate this.

An alternative adjunctive therapeutic strategy, immunosuppression, has long been employed in TB-related meningitis and has proven to be beneficial, with corticosteroids shown to decrease risk of death and drug-induced hepatitis [65, 66]. On the basis of the hypothesis that amelioration of cytokine-mediated pathology might be advantageous in some forms of TB, other combined immunosuppressive-therapeutic strategies have also been advocated [67, 68].

IMPROVED MANAGEMENT AND PREVENTION OF MDR-TB AND XDR-TB

Assigning a name to XDR-TB in 2005 brought it to public attention, accompanied by fears of a return to the preantibiotic era and the specter of untreatable TB [69]. Rapid fatality in people with XDR-TB in Tugela Ferry, South Africa, illustrated the tragic consequences of this infection in the setting of high rates of concurrent HIV infection and nosocomial spread [5].
Although, in sub-Saharan Africa, this burden remains fearsome [6, 7, 70], and although a failure to detect MDR-TB obscures the true scale of this crisis [3], a new picture emerged during 2008 in which XDR-TB cure is a realistic aim and decreases in MDR-TB rates can be achieved, including in resource-limited settings [71–73]. Retrospective cohort studies from diverse locations reported their experiences with MDR-TB and XDR-TB treatment in late 2008 [22, 71, 72, 74], offering an optimistic “potentially favorable perspective for patients” with XDR-TB [22] (eg, cure rates of 66.3% and 60.4% for MDR-TB and XDR-TB, respectively [72]). Level 1 (randomized, controlled trial–derived) evidence is required, as articulated by the Cambridge Declaration on clinical trials for drug-resistant TB [75], but meta-analysis of retrospective observational cohort studies was recently performed. This has identified the WHO recommendations of a treatment duration of ≥18 months after culture conversion and administration of DOT throughout as the factors associated with the greatest chance (almost 70%) of treatment success [76]. Drug susceptibility testing–tailored regimens were associated with nonstatistically significantly better outcomes than were standardized regimens, and inconsistent reporting of HIV status rendered examination of this variable difficult [76]. MDR-TB and XDR-TB treatment programs providing additional measures, such as nutritional and economic assistance for patients, have reported high rates of cure and treatment completion [71, 72], but the relative efficacy of such supports is unknown. High rates of successful treatment outcome may not be widely generalizable; major differences in outcomes are seen in South Africa, even accounting for HIV infection, possibly attributable to low use of quinolones; patient factors (higher use of alcohol, tobacco, and other drugs; malnutrition; and host immunity) and/or M. tuberculosis strain factors (differing virulence) [70], although meta-analysis did not find these factors to have a significant impact on MDR-TB outcome [76].

Even the best outcomes for MDR-TB treatment remain substantially worse than the WHO’s cure target of 85% for drug-susceptible TB, and the pool of undiagnosed MDR-TB and XDR-TB seriously threatens control efforts. Nevertheless, good cure rates provide a notably more positive outlook than the dire reports from only 2–3 years ago. Despite the expenses of MDR-TB treatment, modeling including drug, laboratory, and personnel costs indicates that MDR-TB treatment can be highly cost-effective [77]. There is a clear imperative for managing drug-resistant TB in accordance with current guidelines (eg, in DOTSPlus projects, accessing second-line anti-TB drugs through the Green Light Committee) [26] while not diverting funds away from the core business of basic TB service provision.

An additional cause for optimism is the recent decrease in MDR-TB rates documented in Estonia, Latvia, Hong Kong, and the United States [73]; the next important step is to identify the factors responsible for these successes. Infection control is emerging as a critical focus for MDR-TB and XDR-TB prevention, because they are frequently nosocomially transmitted [4–6, 78], requires renewed enthusiasm for more stringent respiratory infection control. This need not be complicated: modeling of XDR-TB transmission in Tugela Ferry indicated that appropriate use of existing resources (a combination of use of face masks, reduced duration of hospitalization, and a shift to outpatient therapy) could prevent nearly one-third of XDR-TB
cases [79]. Elsewhere, natural ventilation achieved by opening windows and doors was estimated to achieve lower TB transmission than costly, maintenance-requiring mechanical ventilation systems [80]. Recent revisitation of 1950s experimental designs, using exhaust air from a TB ward passed into guinea pig enclosures, demonstrated that low-cost ultraviolet lights prevented 70% of TB infections, and negative air ionization prevented 60% of TB infections [81]. Nosocomial XDR-TB transmission can occur even with good ventilation, limited patients per room, and ultraviolet lamps [4]. Nevertheless, evidence indicates that the ISTC [14] should require an additional 18th standard: that respiratory infection control measures should be implemented in facilities treating patients with newly diagnosed smear-positive TB, especially in areas with a high prevalence of MDR-TB or XDR-TB.

### IMPROVED HIV-TB MANAGEMENT

HIV infection increases the risk of latent TB reactivation 20-fold [82, 83], and in southern Africa, TB is the leading cause of death among people with HIV infection [84]. Identifying HIV infection presents an important TB control opportunity through the use of antiretroviral therapy and isoniazid preventive therapy, both of which reduce the risk of developing active TB and require continued up-scaling [85–88]. Advances in the understanding of management of concurrent HIV infection and TB include improved guidelines on HIV testing in persons with TB (ie, provider-initiated opt-out testing of all people with newly diagnosed TB [7, 89–92]), growing knowledge of optimal antiretroviral therapy regimens to use in combination with antitubercular agents [7, 89–92], and timing of antiretroviral therapy commencement in relation to TB [7, 89, 91, 93–96]. Benefits of isoniazid preventive therapy are also evident [97], although details, such as how to confidently exclude active TB, safety in combination with antiretroviral therapy, and the recommended duration (eg, 12 months, lifelong, or base on CD4 cell count), are areas of ongoing research (http://www.clinicaltrials.gov/NCT00463086).

First-line antiretroviral therapy recommended for persons with concurrent HIV infection and TB by Centers for Disease Control and Prevention and WHO comprises standard-dose efavirenz plus 2 nucleoside reverse-transcriptase inhibitors [91, 92], on the basis of the understanding that the 20% lower serum efavirenz concentration caused by rifampicin remains effective in suppressing HIV replication [98, 99]. Efavirenz may be safer in pregnancy than hitherto realized [92, 100, 101], although it remains a Category D listing. Nevirapine concentrations are more likely to be subtherapeutic in combination with rifampicin [102], and virological failure risk may be higher [103]. Despite this, nevirapine-based antiretroviral therapy is considered a suitable choice for second- or third-line treatment if efavirenz is contraindicated or unavailable and if rifampicins with less potency in inducing cytochrome P450 enzymes (rifapentine or rifabutin) are unavailable (as is generally the case where TB is endemic) [91, 92].

Determining optimal timing for antiretroviral therapy commencement after initiation of TB treatment is challenging because of drug toxicities, interactions, the heavy pill burden and attendant adherence risk, and immune reconstitution inflammatory syndrome [104, 105]. Although immune reconstitution inflammatory syndrome may increase with wider and earlier antiretroviral therapy use [94], it has not been associated with increased mortality, and to date, the risks appear to be outweighed by the benefits of early antiretroviral therapy [7, 106]. Recent cohort studies and preliminary data from one trial indicate that the optimal strategy is to begin antiretroviral therapy during TB treatment and that integration of HIV and TB care offers valuable benefits [93–96]. WHO guidelines recommend commencement of antiretroviral therapy 2–8 weeks after initiation of TB treatment if the CD4 cell count is <350 cells/mm³ or unknown [7, 89, 91]. Additional data, including data from

### Table 3. Summary of Recommendations for Antiretroviral Therapy (ART) in Patients with Concurrent Human Immunodeficiency Virus (HIV) Infection and Tuberculosis (TB)

<table>
<thead>
<tr>
<th>CD4 T cell count</th>
<th>ART recommendation</th>
<th>Timing of ART initiationa</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Drug-susceptible TB</td>
</tr>
<tr>
<td>≤200 cells/mm³</td>
<td>Yes</td>
<td>2–8 weeks after starting TB treatment</td>
</tr>
<tr>
<td>200–350 cells/mm³</td>
<td>Yes</td>
<td>After 8 weeks</td>
</tr>
<tr>
<td>≥350 cells/mm³</td>
<td>No</td>
<td>Reevaluate at 8 weeks and every 6 months thereafter</td>
</tr>
<tr>
<td>Not available</td>
<td>Yes</td>
<td>2–8 weeks after starting TB treatment</td>
</tr>
</tbody>
</table>

**NOTE.** The recommended first-line ART regimen is efavirenz (600 mg daily) plus 2 nucleoside reverse-transcriptase inhibitors, with a rifampicin-based TB regimen given 5–7 times weekly [91, 92]. The rationale for using ART to prevent TB and to improve TB treatment outcome in persons with HIV infection is based on evidence that ART reduces the risk of developing active TB [85, 86] and improves survival when it is started during TB treatment, compared with if it is deferred [93–96], with the benefits of early ART initiation in people with TB outweighing the disadvantage of potential increased occurrence of immune reconstitution disease [7, 106]. Furthermore, ART achieves as-good virological and immunological responses as in people without TB [106].

a From [7, 89, 91].
the SAPIT (Starting Antiretrovirals at Three Points in Tuberculosis Therapy) trial [96], should provide further clarity. On the basis of the evidence summarized above, ISTC Standard 13, which recommends antiretroviral therapy in selected patients with concurrent HIV infection and TB, can be updated to incorporate advice regarding antiretroviral therapy regimen selection and timing of commencement (Figure 1 and Table 3).

TOWARD THE STABILIZATION OF THE “GLOBAL EMERGENCY”

The reported number of TB cases per capita has been decreasing globally since 2003, and funding for TB control has improved, peaking in 2008 at US $3.3 billion [8]. Although the challenges posed by HIV infection and MDR-TB remain formidable worldwide, 3 of the 6 WHO regions (the Americas, the Eastern Mediterranean, and South-East Asia) will meet 2015 targets for reductions in TB case numbers and fatalities [3]. Progress in industrialized countries includes downward trends in TB rates in Indigenous populations. Over the past 20 years, the TB incidence has decreased among Australian Aborigines [107], Canadian First Nations populations [108, 109], and Native Americans [110], attributed to factors that include improvements in case finding and treatment in the early 1990s [108, 109]. These figures do not permit relaxation in TB control but illustrate that, where adequate resources can be directed toward at-risk populations, major benefits can result, and downward trends ought to be maintainable.

New challenges continually arise in TB, but these become opportunities for the recognition and development of innovative TB control strategies. For these advances to translate into mainstream practice in a timely manner, national TB programs need to embrace new evidence, and clinicians in areas where TB is endemic require workable mechanisms to achieve continuing education alongside their heavy workloads. Such mechanisms include the more vigorous promotion of international standards at a national level and timely implementation by practitioners. The 2006 ISTC [14] has proven to be useful in convincing national professional societies and academic institutions to support implementation of internationally recognized standards of TB care [8]. Such has been the rapidity of progress in the past 3 years that we suggest that the ISTC be updated to incorporate the elements summarized in Figure 1. In many parts of the world, such strategies may appear unrealistic. However, even in circumstances of poverty or instability, there can be scope for optimization of resource allocation and mobilization of political will to allow modern TB management to be adopted where it is needed most. Enthusiasm for adopting such changes can be generated by disseminating cost-effective TB success stories. Despite major new challenges, wider incorporation of recent advances, and further optimization of DOTS programs provide grounds for improved TB control.

Acknowledgments

We thank the anonymous reviewers for their very helpful contributions to the final manuscript.

Financial support. The Australian National Health and Medical Research Council.

Potential conflicts of interest. All authors: no conflicts.

References

18. Ramsay A, Yassin MA, Cambanis A, et al. Front-loaded sputum microscopy services: an opportunity to optimise smear-based case de-

<see paper next page>
 Appreciation of what’s new in tuberculosis (TB) requires a perspective of what’s old: Mycobacterium tuberculosis (MTB) and other members of the MTB complex (Table 1) have been infecting humans since antiquity.1,2 Being therefore consummately adapted to life within the human host,3 MTB infects up to one-third of the global population, is characterised by a dormant phase which confounds diagnosis and control, and in 2006 accounted for an estimated 9.2 million new cases and 1.7 million deaths worldwide.4

Australia largely avoided the late 20th century global TB resurgence, which prompted the declaration of TB as a ‘global emergency’ by the World Health Organization (WHO) in 1993. The millennium development goals set a target of reducing prevalence and death rates of TB by 50% by 2015. Although many countries are on track to attain these rates, highest burden countries, notably in sub-Saharan Africa where multidrug resistant TB (MDR-TB) and the overlap of TB and HIV epidemics pose crippling challenges, are not.4

Against this background, Australia in 2006 reported 1201 TB cases, an incidence of 5.8 per 100 000 population,5 with preliminary 2008 figures being similar.6 The national strategic plan for TB control recognises the importance of favourable socioeconomic circumstances; successful post-World War II national TB campaigns; specialised, multidisciplinary, free TB services; and effective premigration screening as contributors to Australia’s low TB rates.7 New challenges faced by Australian practitioners include addressing high rates of TB (~1360/100 000) in Indonesian fishermen entering Northern Territory waters,8 and MDR-TB (25% of cases) in Papua New Guinean nationals seeking treatment in the Torres Strait protected zone.9 This provides a preview of cases Australian practitioners are likely to be more frequently presented with in coming years as the distribution of MDR-TB continues to expand.

Tuberculosis treatment in Australia is conducted by specialists within centralised, coordinated TB services. Failure to manage TB in this fashion is associated with higher risks of treatment failure and emergence of drug resistance.10 However, general practitioners have the key responsibilities of recognising at risk people and coordinating care for affected families. In particular, the most vital contribution GPs can make is to avoid TB diagnostic delay by sending sputum specimens...
for specific examination for acid fast bacilli (AFB) and reviewing chest radiograph findings in at risk people with suspicious cough (>2 weeks or "chronic pneumonia"). This review presents recent developments in TB relevant to Australian general practice. Comprehensive TB management guidelines are provided elsewhere (see Resources).

Who is at risk in Australia?

Tuberculosis requires particular consideration in migrants and refugees from high incidence countries. Approximately 85% of new Australian TB cases occur in people born overseas. Highest global per capita TB rates occur in sub-Saharan Africa, reflected in high TB notification rates in Australians of sub-Saharan origin. Commonest countries of origin of people with TB in Australia (consistent with relative sizes of migrant populations) are India, Vietnam, the Philippines, China and Indonesia. Tuberculosis cases in migrants are usually attributable to reactivation of latent TB infection (LTBI) (often within the first 2 years after migration), with smaller contributions from local transmission within migrant communities, and newly acquired infections after visits home. Negative premigration or arrival TB screening does not therefore negate the possibility of subsequent TB.

Indigenous Australians (in some communities more than others) are at higher risk than non-Indigenous Australian born people (6.6 vs. 0.9 per 100 000). Other commonly identified TB risk factors in Australia are household contact with TB, or residence in a TB high prevalence country for more than 3 months. Returned travellers uncommonly present with TB.

Human immunodeficiency virus confers the greatest single risk for TB, increasing the chance of latent TB reactivation up to 20-fold. In Australia, HIV-TB co-infection is uncommon, but the reporting of HIV status in people with TB, at 37%, is much lower than recommended. A new diagnosis of TB must prompt an offer of HIV testing as per local and international recommendations.

Other forms of immunosuppression also significantly increase the likelihood of LTBI reactivation. In people with LTBI, such as migrants and indigenous people from high TB burden communities, chronic renal failure and high dose corticosteroid use approximately doubles the risk of developing active TB, while TNF-α blockers (eg. infliximab) increase the risk five-fold compared with the comparative population risk of LTBI reactivation.

These at risk groups require screening for LTBI if asymptomatic (see below and Table 2), or investigation for active pulmonary or extra pulmonary TB if symptomatic with a clinically relevant syndrome. People with LTBI at risk of progression to active TB, require LTBI treatment (Table 3). Detailed screening and LTBI treatment guidelines are provided elsewhere.

What’s new in TB diagnostics?

Globally, the commonest method for diagnosing TB remains sputum microscopy only; therefore improved access to inexpensive diagnostics is a priority in under resourced settings. There has been promising progress in the development of such tests, including improved microscopy and culture techniques, and rapid molecular methods for detection of rifampicin and isoniazid resistance gene mutations. These can provide rifampicin/isoniazid susceptibility results within 1–2 days, compared with approximately 42 days using liquid culture media. A reliable point of care test such as antigen detection on blood or sputum would be the ‘holy grail’ of TB diagnostics; such tests are under investigation but not yet commercially available. Nucleic acid amplification tests, including polymerase chain reaction (PCR), have high specificity. Although negative predictive value is low, the possibility of a positive result in smear negative disease can allow more rapid diagnosis while awaiting culture; recently updated Centres for Disease Control (USA) guidelines therefore recommend sputum MTB nucleic acid amplification testing where available and affordable, in cases of suspected, unconfirmed TB, if results would alter management.

Interferon gamma release assays

Of more relevance in Australia are interferon gamma release assays (IGRA), such as QuantiFERON-TB Gold, as potential supplements or alternatives to tuberculin skin testing (TST) for detecting TB exposure. Currently, Australia’s National Tuberculosis Advisory Committee discourages use of these tests in clinical settings pending further research into sensitivity, specificity and cost effectiveness (Table 2), favouring use of clinical history and TST. Like the TST, IGRA detect T-cell responsiveness to MTB antigens, but this is measured on a blood sample as interferon (IFN)-γ production rather than an in vivo skin reaction, and the antigens used to elicit the response are more specific to MTB than those contained in purified protein derivative. Some diagnostic algorithms incorporate these tests.

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Table 1. Mycobacterium TB and other members of the MTB complex

<table>
<thead>
<tr>
<th>Mycobacterium genus</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. tuberculosis</td>
<td>Gram positive, acid fast bacilli. Included MTB complex, pathogenic non-tuberculous mycobacteria (NTM) and M. leprae</td>
</tr>
<tr>
<td>M. bovis</td>
<td>Tuberculosis causing group – MTB complex:</td>
</tr>
<tr>
<td>M. africanum</td>
<td>- M. tuberculosis</td>
</tr>
<tr>
<td>M. bovis Bacillus Calmette Guerin (BCG)</td>
<td>- M. bovis</td>
</tr>
<tr>
<td></td>
<td>- Uncommon: M. microti, M. bovis subspecies capra, M. tuberculosis subspecies canetti, M. pinnipedii</td>
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Table 2. Mycobacterium TB and other members of the MTB complex

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<td>- Uncommon: M. microti, M. bovis subspecies capra, M. tuberculosis subspecies canetti, M. pinnipedii</td>
</tr>
</tbody>
</table>

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Table 3. Detailed screening and LTBI treatment guidelines are provided elsewhere.
as adjuncts to,\textsuperscript{31} or replacements of,\textsuperscript{32,33} TST, hence some familiarity with this test is required.

A ‘take home message’ is that a positive result in either IGRA or TST does not confirm, and a negative test does not exclude, TB exposure.

**What’s new in TB treatment?**

Standard treatment regimens for active and latent disease caused by drug susceptible MTB have not changed in Australia in recent decades (Table 3), however, new developments are discussed below.

**New drugs for TB**

The drought in new TB drug discovery appears to be breaking, with about 30 new agents in the developmental pipeline.\textsuperscript{34,35} A metronidazole related antibiotic, PA-824, has been found to be successful against MTB in vitro and in mouse models\textsuperscript{36} and is now in phase 2 human trials.

Novel agents targeting specific MTB genes are under investigation.\textsuperscript{37} Quinolones, with a well established place in the treatment of MDR-TB, are under investigation for their potential to shorten treatment durations in drug sensitive TB.\textsuperscript{38} Of critical importance is the need to limit misuse of new drugs, eg. use of quinolones as monotherapy in TB misdiagnosed as pneumonia is associated with development of resistance and delayed TB diagnosis.\textsuperscript{39}

**HIV-TB co-infection management**

Traditionally, delayed commencement of antiretroviral treatment (ART) was recommended in HIV-TB co-infection due to overlapping drug toxicities, drug interactions, and the potential for paradoxical worsening of TB pathology due to immune restoration inflammatory syndrome.\textsuperscript{40} However, it is becoming clearer that benefits of early ART outweigh these risks, and that despite rifampicin induced increased clearance of non-nucleoside reverse transcriptase drugs (efavirenz/nevirapine), serum levels of these drugs can remain adequately efficacious to permit their use.\textsuperscript{41} Current guidelines recommend starting ART within 2–8 weeks of TB treatment initiation if CD4 <200, and after 8 weeks if CD4 200–350, preferably using an efavirenz based regimen.\textsuperscript{17,40,41}

The importance of TB screening in people with HIV (eg. using a combination of symptom check, sputum microscopy, TST), and isoniazid preventive therapy if LTBI is identified, are increasingly recognised as critical and underutilised public health measures.\textsuperscript{42}

**MDR-TB management**

Multidrug resistant TB was initially recognised to result from poor treatment programs with low adherence, leading to selection of drug resistance mutations in infecting MTB strains.\textsuperscript{43} However, it

---

Table 2. Advantages and disadvantages of interferon gamma release assays\textsuperscript{29,57–62}

<table>
<thead>
<tr>
<th>IGRA disadvantages compared with tuberculin skin testing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Currently less collective experience with IGRA exists, and there are uncertainties in how to interpret results, eg. when there is discordance between IGRA and TST, discordance between different IGRA types, and when serial IGRA results change (reversion/conversion)</td>
</tr>
<tr>
<td>Requires blood taking, which in infants (a common target group for screening) is more difficult, and often not done by rural/remote health workers and registered nurses</td>
</tr>
<tr>
<td>Loss of patient educational opportunities which are provided when TB nurse performs and reads TST</td>
</tr>
<tr>
<td>IGRA possibly less sensitive</td>
</tr>
<tr>
<td>IGRA test kit more expensive than PPD</td>
</tr>
<tr>
<td>Reversions from positive to negative can occur over time and are difficult to interpret (but usually only occur when the initial reading was low-positive)</td>
</tr>
<tr>
<td>Requires laboratory reagents and staff not generally available in resource limited settings, thereby restricting accessibility in remote settings</td>
</tr>
<tr>
<td>Indeterminate results can occur, eg. due to failures of controls, inadequate cell separation, cross contamination</td>
</tr>
<tr>
<td>Greater incidence of discordance between IGRA and TST in children (usually TST positive, IGRA negative)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>IGRA advantages compared with TST</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGRA only target antigens present in MTB ‘region of difference 1’ (RD11) such as CFP10 and ESAT6, which are not shared by other mycobacteria except M. kansasii, M. marinum and M. szulgai, thereby increasing the specificity of the test</td>
</tr>
<tr>
<td>Unaffected by prior BCG vaccination, for same reason as above (RD1 antigens not present in M. bovis BCG)</td>
</tr>
<tr>
<td>Only requires one clinic attendance if negative</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Disadvantages of both IGRA and TST</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unable to distinguish active from latent disease</td>
</tr>
<tr>
<td>False negatives occur especially in immunosuppression</td>
</tr>
<tr>
<td>No gold standard for LTBI diagnosis, therefore the true sensitivity and specificity of both tests cannot be accurately evaluated</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>A niche for IGRA use?</th>
</tr>
</thead>
<tbody>
<tr>
<td>QFT-G may be strategically used in settings such as previously BCG vaccinated TB contacts with a positive skin test, and BCG vaccinated health care workers in low TB incidence countries who require repeat testing</td>
</tr>
</tbody>
</table>

TST = tuberculin skin test using purified protein derivative (PPD) (also known as Mantoux test)
is increasingly found to be a primary transmitted organism in high MDR burden areas, emphasising the vital importance of infection control. Multidrug resistant TB treatment regimens (Table 3) are prolonged, costly, and associated with significant side effects and adherence difficulties. However, well functioning programs, especially those incorporating the WHO recommendations of ≥18 months treatment duration after culture conversion and directly observed therapy throughout, report cure rates of >60%. Phase 1: includes injectable agent, minimum 6 month duration and at least 4 months past culture conversion Phase 2: after cessation of the injectable agent, continued for at least 18 months after culture conversion

Table 3. Anti-TB agents and treatment strategies

<table>
<thead>
<tr>
<th>Anti-TB agents, WHO classification</th>
<th>Drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1 First line oral antituberculosis agents</td>
<td>Isoniazid, rifampicin, ethambutol, pyrazinamide, rifabutin</td>
</tr>
<tr>
<td>Group 2 Injectable antituberculosis agents</td>
<td>Streptomycin, kanamycin, amikacin, capreomycin</td>
</tr>
<tr>
<td>Group 3 Fluroquinolones</td>
<td>Moxifloxacin, levofloxacin, ofloxacin</td>
</tr>
<tr>
<td>Group 4 Second line oral bacteriostatic antituberculosis agents</td>
<td>Ethionamide, prothionamide, cycloserine, terizidone, para-aminosalicylic acid</td>
</tr>
<tr>
<td>Group 5 Antituberculosis agents with unclear efficacy</td>
<td>Clofazimine, amoxicillin/clavulanate, linezolid, thioacetazone, imipenem/cilastin, high dose isoniazid, clarithromycin</td>
</tr>
</tbody>
</table>

**Standard first line TB treatment**

Rifampicin, isoniazid*, pyrazinamide, ethambutol daily or thrice weekly for 2 month ‘intensive phase’ then rifampicin and isoniazid daily or thrice weekly for 4 month ‘continuation phase’. Dosing (see reference 23)

**Standard LTBI treatment**

Isoniazid* daily 9 months

**MDR-TB treatment strategies**

Two phase regimen using at least five drugs to which the infecting isolate is sensitive. Drugs include remaining first line drugs to which the organism is sensitive (ethambutol and/or pyrazinamide) plus a quinolone (descending order of potency against MTB: moxifloxacin = gatifloxacin > levofloxacin > ofloxacin = ciprofloxacin) plus an injectable agent from Group 2 above, plus others as required. High dose isoniazid may retain efficacy against MDR isolates with low level isoniazid resistance

Phase 1: includes injectable agent, minimum 6 month duration and at least 4 months past culture conversion

Phase 2: after cessation of the injectable agent, continued for at least 18 months after culture conversion

**LTBI treatment where infecting strain is thought to be MDR-TB**

No Level 1 evidence. Two drug regimen tailored to the sensitivity profile of the MTB isolated from the contact may be efficacious, eg. pyrazinamide + ethambutol or a quinolone 6–12 months alternatively, a monitoring approach may be appropriate, especially in children

* Vitamin B6 (pyridoxine) 25 mg/day routinely accompanies isoniazid to reduce the incidence of peripheral neuropathy

** Combination therapy is needed in active but not latent TB, as active TB is characterised by high bacillary numbers, hence the number of bacilli with spontaneously arising resistance mutations at baseline, which can be selected out by inadequate regimens, is much greater than in LTBI

How can GPs care for patients with TB?

Although specialty TB treatment units have the responsibility for reporting and treating TB, managing medication adverse events and ensuring adequate contact tracing (see Resources), GPs are at the forefront of having to suspect TB and LTBI, and integrate TB care with other medical needs of the affected individual and their family.

Avoid diagnostic delay

By considering pulmonary TB in people with symptoms (eg. cough >2–3 weeks, fever ≥3 weeks, loss of >10% body weight) and epidemiological risk factors, GPs have the valuable opportunity to avoid diagnostic delay. Delayed diagnosis aggravates the individual’s morbidity, increases public health risk, and complicates the subsequent contact tracing required.

Key actions to making a timely diagnosis are:

- request AFB examination on sputum in addition to standard microscopy/culture
- improve yield by requesting morning sputum specimens, and arranging 2–3 collections, and
- assiduous follow up of chest radiography.

As a negative smear does not exclude pulmonary TB, clinical suspicion in a person from a TB endemic country warrants referral to TB services (see Resources), regardless of AFB sputum smear result.

Infection control

General practices require a policy for ensuring adequate respiratory precautions for people with suspected or confirmed infectious pulmonary TB attending their practice, or whom they care for in nursing homes, prisons or other institutions, including avoidance of communal areas and use of face masks.
Avoid drug interactions
Rifampicin is a potent inhibitor of cytochrome P450 isoenzymes, especially CYP3A4 and CYP2CB/9, and therefore great attention to other medications metabolised by these enzymes, especially anticonvulsants, oral contraceptives and warfarin, is required.

Nutrition
Malnutrition contributes to TB risk and complicates active TB.\textsuperscript{50} Specific micronutrients with immunological functions such as vitamin D may be of particular importance,\textsuperscript{51,52} but trial outcomes examining use of these agents as supplements in TB treatment are pending. Food scarcity is obviously less problematic in Australia than in high TB burden countries, but nutritional optimisation and replacement of deficient nutrients in individuals with TB is in keeping with current WHO recommendations.\textsuperscript{53}

Smoking cessation
Active and passive smoking are associated with elevated TB risk and worse outcomes from TB infection.\textsuperscript{54} Smoking cessation is therefore an important potential intervention area, although the impact of quitting after diagnosis on individual patient outcomes remains to be determined.

Psychological support and adherence help
Psychological and practical supports are key requirements of TB care. Tuberculosis remains deeply stigmatised in many societies, and confinement in respiratory isolation can be traumatising.\textsuperscript{55} A diagnosis of TB can therefore require management of negative psychological effects. The prolonged duration of therapy requires assiduous measures to promote adherence. The most widely used of these is the WHO sanctioned ‘DOTS’ (directly observed therapy – short course). Other adherence promoting measures include addressing financial costs of leave from work by assisting in accessing short term disability pensions, addressing any problematic beliefs about TB including those to do with law and migration status, and providing ongoing education about the need to continue treatment even after symptom resolution.\textsuperscript{56}

Conclusion
Important new developments in TB diagnosis and treatment have occurred in recent years. These include the availability of new diagnostic tests, updated guidelines on managing HIV-TB co-infection, new data showing better prognoses for people with MDR-TB/XDR-TB, and renewed emphasis on the importance of infection control. Australia remains in the fortunate position of having very low TB rates by global standards, low drug resistance (although increasing cases are projected), infrequent cases of HIV co-infection, and access to MTB culture and susceptibility testing. Maintaining this position despite the high global burden of TB requires ongoing timely TB screening, a low threshold for considering the diagnosis of active TB in people at risk, and psychosocial support to assist adherence to treatment.

Resources
Australian state and territory TB contacts
- Australian Capital Territory Tuberculosis Service, c/o Thoracic Unit, The Canberra Hospital. Phone 02 6244 2066
- New South Wales Tuberculosis Program, NSW Department of Health. Phone 02 9391 9277
- Northern Territory Tuberculosis Unit, Centre for Disease Control. Phone 08 8922 8804
- Queensland Tuberculosis Control Centre. Phone 07 3896 3939
- South Australian Tuberculosis Services, Royal Adelaide Hospital Chest Clinic. Phone 08 8222 4867
- Tasmanian Tuberculosis Services, Southern Region – Respiratory Unit, Royal Hobart Hospital, phone 03 6222 7353; Northern Region – Respiratory Unit, Launceston General Hospital, phone 03 6348 7708; North-Western Region – Chest Clinic, Community Nursing, phone 03 6421 7700
- Victorian Tuberculosis Control Section, Department of Human Services. Phone 03 9096 5110 or 1300 651 160
- Western Australian Tuberculosis Control Program, Perth Chest Clinic. Phone 08 9219 3222.

Selected guidelines and resources
- Stop TB Partnership. Available at www.stoptb.org/.

Fact sheets for patients

Conflict of interest: none declared.

Acknowledgments
Anna Ralph is supported by the National Health and Medical Research Council (NHMRC) and a Royal Australasian College of Physicians – Covance Award. Paul Kelly is supported by NHMRC. We thank the members of the National TB Advisory Committee for providing the contacts listed in the resources table.

References


3 Introduction II: Adjunctive Treatment in TB

Problems with current standard TB treatment regimens, rising drug resistance and the challenges posed by HIV-TB co-infection are driving a need for novel therapeutic approaches in TB. Acknowledging this, the investigation of adjunctive immunotherapies (agents given in addition to antibiotics to beneficially modulate immune responses and thereby improve outcomes), was nominated by the UNICEF/UNDP/World Bank/WHO Special Programme on Research and Training in Tropical Diseases in 2006 as one of six priority TB research areas. In this chapter, I present the rationale for investigating candidate adjunctive immunotherapies in TB, specifically, L-arginine and vitamin D.

3.1 WHY ARE NEW TREATMENT APPROACHES NEEDED?

**Problems with the standard TB treatment regimen**

Progressive refinement of TB treatment regimens between the 1940s and 1970s culminated in the finding that the addition of rifampicin, by accelerating culture conversion rates, permitted treatment to be reduced from 18 to 6-9 months. The resulting current 6-month ‘short-course’ regimen for drug-susceptible MTB infection comprises rifampicin, isoniazid, pyrazinamide and ethambutol for 2 months (intensive phase) then rifampicin, and isoniazid for 4 months (continuation phase). This complex and protracted regimen has high risks for adherence difficulty. Additional problems with the regimen are drug interactions (due to rifampicin’s strong induction of cytochrome P450), antibiotic resistance, and toxicity. Thus a key goal in contemporary TB research is to work towards shorter treatment courses, permitting more rapid disease resolution and earlier cure. Achieving these goals could have important clinical, public health and economic benefits including a faster return to employment or school, and diminution of residual respiratory impairment. Exciting recent developments in this field include the findings that moxifloxacin as an ethambutol substitute accelerates culture conversion in infection with drug-susceptible MTB, and the diarylquinolone
TMC207 (targeting ATP synthase) significantly reduces time to culture conversion when added to MDR-TB treatment regimens.\textsuperscript{13} Ongoing investigation of rifamycins, including higher doses of rifampicin with the potential to accelerate treatment response,\textsuperscript{14} are also under investigation.\textsuperscript{15}

\textit{Adherence}

Adherence to TB treatment is compromised by many factors, which have been summarised by Munro et al\textsuperscript{16} as structural (e.g. financial burden including work absenteeism during treatment, cost of regular transport to the clinic, distance between patient’s home and clinic), personal (e.g. attitudes towards illness and treatment, lack of belief in tablet efficacy, poor patient-staff relations), social (family / community responses including stigmatisation of the TB sufferer), and health service factors (the lengthy treatment duration, pill burden and adverse effects).\textsuperscript{16-18}

The most widely used method of promoting adherence is the use of directly observed therapy (DOTS). Introduction of DOTS has been associated with documented reductions in TB prevalence\textsuperscript{19, 20} (although meta-analysis did not find clinic-based DOT to be superior to routine self-administration, or supervision at home by a family or community member, in achieving TB cure\textsuperscript{21}).

Patient incentives such as a cash deposit paid at treatment commencement are used widely, including at the field research site at which the AVDAPT study is based. Pill burdens are reduced by use of fixed-dose combination (FDC) therapy, but people >55kg still require four FDC tablets per dose, FDC may only be intermittently unavailable (as at our field research site), and FDC is not suitable when adverse reactions require tailored regimens.

Rates of successful treatment completion vary according to the resources available for supporting DOTS or other adherence-promoting measures.\textsuperscript{16} Examples of reported treatment default rates are 20-50% in the US in the 1980s,\textsuperscript{22} and 28% in the Philippines in the 1990s.\textsuperscript{23} Treatment completion rates were as low as 12% at the RSMM community hospital near the Timika TB clinic in 2007 (personal communication, Drs
Frans Thio, Director, Rumah Sakit Umum Daerah [Regional General Hospital], Timika, Indonesia).

**Drug interactions**

Rifamycins vary in the potency with which they induce the cytochrome P450 enzyme system; rifampin is the most potent, strongly inducing CYP3A4 and CYP2C8/9 isozymes and, to a lesser extent, CYP2C19 and CYPD6 isozymes. However, alternative rifamycins are not widely available at low cost in high TB burden countries. Therefore drug interactions are an important consideration; serum levels and therefore efficacy is significantly diminished for many potentially co-prescribed medications (e.g. oral contraceptives, anticonvulsants). Most problematic are potential interactions with antiretroviral therapy (ART). However recent data in fact indicate that while protease inhibitors are definitely contraindicated, adequate serum levels of efavirenz are maintained in combination with rifampicin, and the reduction in serum nevirapine is not as profound as previously supposed; thus there can be greater confidence in following guidelines recommencing early commencement of ART in people being treated for TB.8,24

**Toxicity**

Although adverse events to antituberculosis drugs requiring treatment modification are thought to occur in less than 10% of people (e.g. ranging from 2.3% in those ≤19 years to 8.4% for those ≥60 years in one study25), milder adverse events such as pyrazinamide-induced arthralgia affect up to 40% of people,26 and can be an important cause of morbidity and non-adherence.27

**Drug resistance**

The above-mentioned difficulties in achieving TB course-completion, particularly where structural and health service factors impede careful adherence, promote the development of acquired and amplified resistance in *Mycobacterium tuberculosis* (MTB).28 Transmission of MDR-TB, rather than resistance-development during
treatment, is now increasingly common. Unfortunately resistance mutations do not generally appear to be associated with any significant MTB fitness cost. WHO’s ‘Anti-tuberculosis drug resistance in the world’ reports document the growing problem of global MDR-TB, with the tally of countries reporting cases of XDR-TB inexorably rising.

Treatment regimens for MDR or XDR-TB are more complex, toxic, expensive and prolonged than the standard TB regimen, creating major challenges for health systems and added difficulties for patient adherence. However, comprehensive treatment of MDR and XDR-TB can achieve good outcomes, as reviewed above.

As rates of MDR and XDR-TB rise and their geographical distribution expands, the need for new anti-tuberculous antibiotics and / or novel adjunctive therapies with different mechanism of resistance development becomes increasingly evident.

**HIV-TB co-infection**

As reviewed above, the HIV pandemic is a major factor responsible for failures to control TB into the 21st century. Pulmonary TB is a ubiquitous opportunistic infection in HIV infection, even in early stages before immune deficiency as defined by CD4 count becomes evident. Combating high TB rates among HIV positive people, even before they are eligible for antiretroviral therapy (i.e. CD4 count <200 in resource-limited settings), requires broad approaches including research into new anti-tuberculous chemotherapeutic agents.

### 3.2 ADJUNCTIVE THERAPIES

**ADJUNCTIVE IMMUNOTHERAPEUTIC STRATEGIES**

These points provide a clear rationale for the need for new approaches to TB treatment. While new antibiotics (reviewed elsewhere) are arguably of greatest importance, the investigation of potential adjunctive immunotherapies is recognised as an additional important priority. Immunotherapy strategies in TB have been reviewed by other authors and key points are summarised in the paper reproduced below. The two
contrasting strategies of immune modulation to improve TB treatment outcomes can be broadly categorised as:

- Promotion of the host’s innate or Th1 cell-mediated anti-mycobacterial immune responses;

- Mitigation of immunopathology-mediated tissue damage due to excessive inflammatory responses (immunosuppression).

L-arginine and vitamin D have anti-mycobacterial properties which render them suitable candidate adjunctive immunotherapies in TB, thus bringing together the fields of adjunctive immunotherapy and nutrition in TB.

**Nutritional Supplementation in TB**

There is a clear bi-directional relationship between malnutrition and TB, with underweight individuals having higher risk of developing active TB, and TB causing cachexia. However the corollary that improved nutrition decreases TB risk, or improves outcomes in active TB, is difficult to demonstrate. Nevertheless, nutritional optimisation is recommended by the WHO, especially in the management of drug resistant TB, and is a feature of successful comprehensive treatment programs.

There has long been interest in the use of nutritional agents to improve immune responses to a variety of infections. Some investigations have shown disappointing lack of efficacy (such as vitamin C in common cold), while others have shown important benefits (such as vitamin A in measles and zinc in diarrhoeal disease). Supplementation of specific micronutrients in TB may be required not only to replenish the deficiency states which accompany active TB, but because of the roles certain micronutrients have in mediating anti-mycobacterial immune responses. As well as considering which micronutrients would be most efficacious on the basis of their immunological mechanisms of action, other important questions include whether people with active or latent TB would be better targets for nutritional supplementation, whether micronutrients at achievable doses can offer sufficient potency to significantly influence TB treatment outcome, whether supplementation is of any benefit if given to non-deficient people, and whether a ‘magic bullet’ micronutrient tablet approach is preferable to whole food supplementation. To date, a large number of randomised trials
of nutrition in TB have sought to answer these questions, including investigation of individual supplements, multi-micronutrients of differing combinations and doses, and whole food.

Meta-analysis of nutritional supplements for people being treated for active TB recently identified some evidence for improved weight gain with energy supplements and some combinations of zinc with other micronutrients.\(^46\) For example, a trial investigating the effect of a high-energy food supplement on anthropometric and quality of life outcomes found improved weight and grip strength in those receiving the supplement.\(^47\) A Tanzanian study in almost 500 people found a small but significant increase in weight in people assigned to supplementation with vitamins A, B1, B2, B12, C, E, D3, folic acid, niacin, selenium, copper and zinc, but no effect on culture conversion.\(^48\) In a larger trial evaluating mortality as the outcome measure, no impact was found from supplementation of vitamins A, B1, B2, B6, B12, C, D, E, niacin, folate, zinc, iodine and selenium in HIV positive or negative TB patients.\(^49\) A more recent trial of micronutrient combination therapy (vitamins A, B1, B2, B6, B12, C, E, folic acid, niacin and selenium) found decreased risk of ‘TB recurrence’ in the supplemented group (but ‘recurrence’ was somewhat unusually defined as reversion to smear positivity during treatment) with a greater effect in HIV-infected patients.\(^50\) Supplementation in this study also increased CD3 and CD4 T cell counts. A wholefood trial comprising a nutritious daily meal was associated with significant improvements in weight gain in the intervention arm in pulmonary TB patients in Timor Leste.\(^51\) In summary, certain nutritional supplements may improve weight gain in people with TB, but no convincing impacts on bacteriological outcomes have been identified in trials to date.

### 3.3 ARGinine AND Vitamin D AS NOVEL POTENTIAL ADJUNCTIVE TREATMENTS IN TB

**L-arginine hydrochloride**

L-arginine is a conditionally essential amino acid found in a variety of food sources including nuts, meat and dairy products. Normal dietary intake in well-nourished people provides around 4 to 5g L-arginine per day.\(^52\) L-arginine is the precursor of nitric oxide (NO), a molecule with key vascular and immunological functions, and L-arginine also has T cell immunomodulatory effects.\(^38\)
Use of L-arginine in human trials

Clinical trials of L-arginine in oral, intravenous or inhaled form have been performed examining endovascular and immunological functions, including in cardiovascular conditions, pulmonary hypertension in sickle cell disease, cystic fibrosis, and as an adjunct to vaccination.27-37 Cardiovascular studies have found that at doses of 9 to 30 g daily orally or intravenously, L-arginine can significantly improve coronary blood flow and angina symptoms in patients with non-obstructive coronary artery disease,53 acutely reduce blood pressure,54 and improve claudication distance in patients with peripheral vascular disease.55 In people with severe falciparum malaria, who have lower serum L-arginine than healthy controls, administration of intravenous L-arginine 3 to 12 g has been shown to improve endovascular function, as measured using reactive hyperemia – peripheral arterial tonometry, and to increase exhaled NO by 55%.56 Use of 30g L-arginine daily in people with breast cancer was found to be associated with enhanced host defences, including lymphocyte mitogenic responses and NK cell activity 57.

L-arginine Pharmacokinetics

Healthy adult plasma arginine levels range from 20 to 180 μmol/L.53 Orally administered L-arginine-hydrochloride bioavailability ranges from 21% after a 10g oral dose in one study58 to 68% after a 6g dose in another.54 Peak plasma concentrations (Cmax) of 310 ± 152 μmol/L occur at approximately 90 minutes after a 6g oral dose (tmax), and half life is reported to be 77.5 ± 9.3 minutes.54

VITAMIN D₃ (CHOLECALCIFEROL)

Vitamin D₃ is generated in the skin from 7-dehydrocholesterol on exposure to Ultraviolet (UV) B light and is metabolised in the liver to 25-hydroxyvitamin D₃ (cholecalciferol). Dietary sources (oily fish, fortified foods such as margarine) also provide a small contribution to total body vitamin D. 25-hydroxyvitamin D₃ is converted by the enzyme 1α-hydroxylase to the biologically active steroid hormone, 1,25-dihydroxyvitaminD₃ (cholecalciferol or calcitriol, abbreviated 1,25(OH)₂D₃). It is now recognised that the gene encoding 1α-hydroxylase, CPY27B, is expressed widely
by human cells, and that 1,25(OH)₂D₃ has diverse roles in most organs including anti-proliferative and immunomodulatory effects.⁵⁹

**Vitamin D deficiency and its measurement**

Traditional methods of measuring serum vitamin D (e.g. radioimmunoassay, chemiluminescent assay) tend to lack reliability.⁶⁰, ⁶¹ A more robust and reproducible method using isotope-dilution liquid chromatography-tandem mass spectrometry has been described,⁶² and is now available in Australia.⁶³

There have been calls to revise ‘normal’ reference ranges for serum vitamin D, using the vitamin D level needed for maximal suppression of parathyroid hormone to signify sufficiency, rather than using population measures to develop normal reference ranges. Thus, some enthusiasts suggest that levels as high as 80 nmol/L should be considered the lower level of normal for serum vitamin D,⁶⁴ while others accept 50 nmol/L.⁶⁵ Australian guidelines define mild deficiency as being 25 - 50 nmol/L, moderate as 12.5 – 25 nmol/L, and severe as <12.5 nmol/L.⁶⁶

Even using standard vitamin D reference ranges, high rates of vitamin D insufficiency and deficiency are being recognised globally, likely a consequence of modern lifestyles (indoor living and clothing). This ‘pandemic’⁵⁹ of vitamin D deficiency is not confined to high latitudes but affects all surveyed populations, including in tropical / equatorial and sunny regions in peoples with varying degrees of skin melanisation.⁶⁷-⁶⁹

**Associations of adverse health outcomes with vitamin D deficiency**

Coupled with the appreciation of the pandemic nature of vitamin D deficiency has been the discovery of the pleiotropic actions of 1,25(OH)₂D₃, thus generating many recent observational and some interventional studies demonstrating an association between vitamin D deficiency and a broad range of adverse health outcomes, beyond rickets and osteomalacia. These include neoplastic disease, auto-immune diseases, cardiovascular disease, diabetes, mental health problems, and infections.⁷⁰-⁷⁶ Meta-analysis has also found an association between vitamin D deficiency and all-cause mortality.⁷⁷ The wide-ranging actions of vitamin D, reviewed by Holick⁵⁹ and summarised in Box 3.1, partly explain these associations. The need for either adequate UV exposure, or oral vitamin D intake or supplementation, to avoid vitamin D deficiency-related diseases has led to
calls for reconsideration of public health messages advising sun avoidance to reduce skin cancer risk.\textsuperscript{78}

\textit{Vitamin D Pharmacokinetics}

Vitamin D replacement can be given as large intermittent bolus doses, a concept embraced in the AVDAPT study design (see Chapter 5). The volume of distribution of vitamin D is large since it is fat soluble, necessitating large doses before any change in plasma vitamin D level is detectable. Intermittent depot vitamin D dosing known as ‘stoss’ therapy is among the recommendations for restoration of vitamin D deficiency.\textsuperscript{79} A recent study of cholecalciferol pharmacokinetics in 30 healthy adults with normal baseline vitamin D concentration found that such dosing was safe, resulting in no cases of vitamin D intoxication and no hypercalcaemia.\textsuperscript{80}

\textbf{Box 3.1: Actions of Vitamin D}

- **Calcium and bone**
  - Maintenance of normal serum calcium and phosphorus levels.
  - Control of bone mineralisation and turnover.
- **Anti-proliferative effects**
  - $1,25(\text{OH})_2\text{D}_3$ controls genes in healthy and malignant cells involved in cellular proliferation, differentiation and apoptosis. This is thought to explain the beneficial effect of vitamin D in the hyper-proliferative skin disease psoriasis, and to explain the association between vitamin D deficiency and increased cancer risk.
- **Immunomodulation**
  - Suppression of Th1 activity: anti-autoimmune effect (type 1 diabetes, multiple sclerosis, rheumatoid arthritis, Crohn’s disease).
  - Promotion of macrophage activity: upregulation of genes leading to cathelicidin gene upregulation and the generation of antimicrobial peptide LL-37.
- **Cardiovascular effects**
  - $1,25(\text{OH})_2\text{D}_3$ reduces renin production, decreases proliferation of myocardial and vascular smooth muscle cells, decreases LDL, increases HDL, and enhances insulin production.
**USE OF L-ARGININE AND VITAMIN D IN TB**

The antimycobacterial immunomodulatory properties of L-arginine and vitamin D, and the rationale for investigating their use as adjunctive immunotherapies in tuberculosis, is presented in the paper reproduced below.\(^{38}\)

Further relevant studies pertaining to L-arginine or vitamin D in TB which have appeared since publication of this paper are discussed in the section ‘Literature update’ below.

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<see paper next page>
L-arginine and vitamin D: novel adjunctive immunotherapies in tuberculosis

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Worsening drug resistance and the need for prolonged treatment in tuberculosis (TB) require innovative solutions including investigation of inexpensive, safe adjunctive immunotherapies. L-arginine, the precursor of nitric oxide, and vitamin D recently have elucidated mycobactericidal and immunomodulatory actions against TB and are deficient in people with TB. We review the potential of these agents as adjunctive TB immunotherapies and explore how comparative clinical trials might help clarify their relative importance in the human TB immune response. By enhancing mycobacterial killing in macrophages, L-arginine and vitamin D might have the potential to enable shorter duration of treatment, reduced infectivity and improved response in drug-resistant TB.

Nitric oxide and vitamin D as adjunctive tuberculosis immunotherapies

Mycobacterium tuberculosis (MTB) is one of the most successful human pathogens, infecting an estimated one-third of the human population for a lifelong duration and accounting for the greatest number of deaths from a curable infectious disease [1,2]. Shortening the duration of tuberculosis (TB) treatment by using strategies such as revised regimens or modes of delivery of existing drugs, the development of new antimicrobial agents and successful adjunctive immunotherapies could profoundly impact TB cure rates [3–5]. Here, we examine two agents whose therapeutic potential as adjunctive TB immunotherapies derives from their predominant (although not exclusive) enhancement of macrophage antituberculous responses.

The L-arginine–nitric oxide (NO) and vitamin D pathways comprise two parallel systems utilized by macrophages to kill MTB, an example of redundancy as an independent pathway, namely via modulation of T-cell function through L-arginine’s effect on the expression of CD3ζ, a component of the T-cell receptor (TCR) [8]. The role of the vitamin D3 pathway in mycobacterial killing within human macrophages has been carefully elucidated in recent studies [6,9]. The importance of vitamin D3 in human host resistance TB has been suspected since the late nineteenth century [10,11]. Now, expanding recognition of the association between vitamin D deficiency and TB risk and of the pleiotropic immunomodulatory functions of vitamin D3 provides biological mechanisms for this supposition and has lead to calls for the clinical evaluation of vitamin D as an adjunctive therapy in the treatment of active tuberculosis [6,12]. In this review we explore the potential clinical application of both L-arginine and vitamin D as adjunctive treatments in TB.

Adjunctive therapy in tuberculosis

Immunosuppressants and proimmune mediators (i.e. enhancers of immunological function) both have roles as adjuncts to TB treatment. Corticosteroids, possibly by suppressing tumour necrosis factor-α (TNF-α) and other proinflammatory cytokines, can ameliorate excessive host inflammatory T-cell responses and subsequent organ damage and have the best recognized role as TB adjunctive treatments [13]. Positive results have been obtained in some trials with thalidomide, also acting via TNF-α [14]. Deliberate therapeutic disruption of granulomata using high-dose corticosteroids or etanercept, combined with standard antituberculous drugs, has been suggested as a novel means of accelerating TB cure [15].

By contrast interest in proimmune mediators as adjunctive immunotherapies is growing and could become increasingly important as drug-resistant TB becomes more prevalent [3,4,16]. Enhancement of CD8+ cytotoxic T-lymphocyte responses and suppression of T helper 2 (Th2) processes appear promising in animal immunotherapy studies [3]. Human data from mostly small trials of Th1 macrophages [7]. Although early reports cast doubt on the importance of the L-arginine–NO pathway in human macrophage killing of MTB, we review here the evidence for an important functional role in humans. Within the past 2 years, evidence has emerged in humans for an immunomodulatory role of L-arginine via an NO-independent pathway, namely via modulation of T-cell function through L-arginine’s effect on the expression of CD3ζ, a component of the T-cell receptor (TCR) [8]. The role of the vitamin D3 pathway in mycobacterial killing within human macrophages has been carefully elucidated in recent studies [6,9]. The importance of vitamin D3 in human host resistance TB has been suspected since the late nineteenth century [10,11]. Now, expanding recognition of the association between vitamin D deficiency and TB risk and of the pleiotropic immunomodulatory functions of vitamin D3 provides biological mechanisms for this supposition and has lead to calls for the clinical evaluation of vitamin D as an adjunctive therapy in the treatment of active tuberculosis [6,12]. In this review we explore the potential clinical application of both L-arginine and vitamin D as adjunctive treatments in TB.

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arginosuccinate lyase (ASL) [23]. Arginine also can be
arginine turnover. Citrulline is synthesized in the gut and
protein turnover contributes the majority of endogenous
5%–15% of endogenous arginine flux [23]. Whole-body
[23]. In adults
body protein degradation plus synthesis from citrulline)
exogenous sources (diet) and endogenous sources (whole-
identified rapid-response receptors, which might be
VDR, resulting in genomic responses, or via more recently
discovered ‘adjunctive therapy’ with vitamin A, zinc or multivitamins
or high-energy and protein food supplements have shown
disappointingly modest benefits [20–22].

**L-arginine and vitamin D physiology**

**L-arginine**

L-arginine is a conditionally essential (semiessential)
aming acid in humans. *In vivo*, arginine derives from
exogenous sources (diet) and endogenous sources (whole-
body protein degradation plus synthesis from citrulline)
[23]. In adults *de novo* arginine synthesis accounts for only
5%–15% of endogenous arginine flux [23]. Whole-body protein
turnover contributes the majority of endogenous
arginine turnover. Citrulline is synthesized in the gut and
then converted to arginine in the kidneys via actions of the
cytosolic enzymes argininosuccinate synthetase (ASS) and
argininosuccinate lyase (ASL) [23]. Arginine also can be
produced in cells actively producing NO via the citrulline–
NO cycle (Figure 1). During hypercatabolic states such as
infection or trauma, arginine synthesis can no longer
match catalysis and an exogenous source of arginine
becomes essential to maintain the extracellular
concentrations required for optimum NO synthesis.
Hypoorarginemia has been demonstrated in people with
pulmonary TB [8], malaria [24], sepsis [25] and pregnancy
[26]. Hypoargininemia in people with active TB, due to
excessive arginase activity, might be difficult to overcome
from dietary means alone due to disease-related anorexia.

**Vitamin D**

Cholecalciferol (vitamin D₃ or 25(OH)D₃) is converted into
the biologically active hormone 1,25-dihydroxyvitamin D₃
(1,25(OH)₂D₃) by 1α-hydroxylase, encoded by the gene
*CYP27b1*. Exogenous determinants of vitamin D₃ levels
include ultraviolet light B exposure, dietary intake and TB
drugs themselves, with isoniazid inhibiting and rifampicin
inducing cytochrome 3A4, a vitamin D hydroxylase [27].
Endogenous determinants include genetic factors [e.g., skin
pigmentation and vitamin D receptor (VDR)
polymorphisms], vitamin-D-binding protein (DBP)
concentration and macrophage activation. Activated
macrophages generate active vitamin D₃ due to
upregulation of *CYP27b1* [28] (Figure 2). Vitamin D₃
actions are mediated either by ligation with the nuclear
VDR, resulting in genomic responses, or via more recently
identified rapid-response receptors, which might be
associated with the plasma membrane [29]. Vitamin D is
transported by the polymorphic, multifunctional DBP. As
well as carrying vitamin D (which occupies only ~5% of
binding sites), DBP transports other immunomodulatory
molecules and when deglycosylated becomes DBP-
macrophage-activating factor (DBP-MAF), a potent
stimulator of macrophages and osteoclasts [30]. Whether
altered DBP kinetics might modulate vitamin D handling
in the setting of macrophage activation in TB is worthy of
speculation.

**Epidemiology**

Accumulating evidence of an association between low
serum vitamin D levels in individuals, certain ethnic
groups, migrants and others, and a higher risk of active
TB [6,31–35] now has been confirmed in a recent meta-
analysis [36]. Hypovitaminosis D in people with active TB
might be attributable to factors including genetic
determinants; inadequate cutaneous synthesis due to
environmental conditions or medically or socially imposed
isolation; or reduced dietary intake due to preference,
availability or TB-induced anorexia.

**Genetics**

VDR polymorphisms have been investigated as possible
genetic determinants of TB risk. Meta-analysis has been
inconclusive [37], but findings to date suggest that the
*TagI* tt polymorphism might be associated with a shift
towards beneficial Th1 responses and at least one t allele
might be associated with better MTB-directed cell-
mediated immune (CMI) responses [38]. NOS2A
polymorphisms of the gene encoding NOS2 (nitric oxide
synthase 2) (Figure 1) also have been investigated. In one
study long repeats of a microsatellite in the promoter
region of the NOS2A gene were over-represented in Papua
Columbians with TB compared with controls [39].
Interpretation of findings can be difficult because many
polymorphisms investigated have unknown functions.
Furthermore, increasing recognition of non-VDR-mediated
vitamin-D-signaling pathways (via membrane-associated,
rapid-response receptors), especially within the immune
system, indicate that VDR polymorphisms cannot be
expected to completely explain genetic differences in
vitamin D metabolism.

**Immunomodulatory role of L-arginine in TB**

**Nitric oxide as a mycobactericidal mediator**

L-arginine-derived NO plays a key role in the innate
immune system and, specifically, in the defense against
mycobacteria [7,40] (Table 1). NO kills MTB by direct
bacterial cell damage through deamination of bacterial
DNA, proteins and lipids and by induction of apoptosis of
TB-harbouring macrophages [41]. NO is noted to be
capable of killing TB bacilli in vitro with a molar potency
comparable to that of antibiotics [42]. Early *in vitro*
studies of human monocyte and macrophage responses to
MTB failed to demonstrate a role for NO due to
methodological problems [41]. Despite initial doubts, there
is now compelling evidence that human macrophages and
monocytes are capable of high level NOS2 expression and
inducing NO production in vivo [43], including in TB [44–45], and subsequent increased capacity for MTB killing (Table 1).

**Induction of MTB dormancy gene expression by NO**

NO at a low (nontoxic) concentration appears capable of driving MTB to enter a dormant phase of inhibited metabolism and consequent chemosensitivity, which might equate with latency (Ref. [46] and reviewed in Refs [47,48]). Thus, although high NO concentrations are mycobacterial, based on this literature low NO states resulting from L-arginine deficiency or inadequate NOS2 expression could be doubly disadvantageous to the host by contributing to the development of latent TB infection.

**Mycobacterial resistance to NO**

Emphasizing the fundamental role of NO in MTB immune responses, MTB has developed specific strategies to mitigate NO-mediated microorganism toxicity [47]. MTB strains also demonstrate variable degrees of susceptibility to NO. One TB strain accounting for a large proportion of new drug-susceptible TB cases in New York in the early 1990s was relatively resistant to reactive nitrogen intermediates (RNI) compared with other circulating MTB strains [49]. Although this strain appeared to have an advantage allowing it to account for more cases than expected, the disease severity in affected patients was not described, whereas in guinea pigs, at least, RNI-resistance among MTB isolates does correlate with virulence [50].

**L-arginine and TCR function**

L-arginine has a further important immunomodulatory role independent of NO through its effects on the CD3ζ component of the TCR. CD3ζ, expressed on the surface of natural killer (NK), CD4 and CD8 T cells, plays a central role in initiation of signal transduction leading to T-cell activation, proliferation and cytokine secretion [51].

The causal pathway between increased arginase activity leading to L-arginine depletion and decreased CD3ζ expression leading to relative T-cell hyporesponsiveness is increasingly understood. Peripheral blood mononuclear cells from people with pulmonary TB have elevated levels of arginase, leading to L-arginine catabolism and hypoargininemia; their consequently decreased CD3ζ expression normalises with successful TB treatment [8]. Separate studies have shown that restoration of extracellular L-arginine ex vivo (in L-arginine-deficient human plasmacytoid cell suspensions and leukaemic cells) leads to CD3ζ re-expression and recovery of T-cell proliferation [26,52]. These data collectively provide important evidence that reduced L-arginine is associated with impaired CD3ζ expression and relative T-cell hyporesponsiveness.

To date there have been no clinical studies in humans testing whether L-arginine supplementation can reverse CD3ζ-associated in vivo T-cell dysfunction, although L-arginine supplementation in normal subjects and patients with breast cancer enhanced NK and lymphocyte-activated NK cell activity [53].

**Immunomodulatory role of vitamin D in TB**

Pre-antibiotic era efforts at TB treatment (Box 1) might have exploited immunomodulatory effects of vitamin D. These have been the subject of recent reviews both in TB [12,54] and the broader immunological context [55].

**Role of activated macrophages**

Macrophages are important sites of vitamin D metabolism in patients with TB. Hypercalcemia occurs in a minority of people with TB and other granulomatous diseases due to CYP27B1 upregulation in activated macrophages [28]. Despite epidemiological data showing low vitamin D levels in TB patients on the whole [36], in selected individuals vitamin D3 levels can reach high concentrations in the vicinity of the granuloma, with spillover into the systemic circulation resulting in hypercalcemia in up to 25% of TB patients, although this uncommonly reaches clinically significant levels [56].

Before the specific role of 1,25(OH)2D3 in the immune response to TB was recognized, speculation regarding the functional rationale for its production by activated macrophages included (i) macrophage-derived vitamin D3 acting as a local downregulator of T-cell activity [57], (ii) a modulator of monocyte-macrophage differentiation [58] or (iii) vitamin D3 attracting DBP to the vicinity of the granuloma, with DBP-MAF further escalating macrophage activation [30]. All of these possibilities might still have relevance, but the mechanism and rationale for CYP27B1 upregulation is now clearer [6], as detailed below.

**Mechanism of vitamin D activity against MTB in cultured human cells**

The inhibitory effect of vitamin D3 on MTB in cultured human monocytes or macrophage cell lines has been recognized since 1986 and replicated in many subsequent studies [6,9,59–62]. Macrophage activation via the TLR (toll-like receptor) 1/2 pathway initiates a cascade of events culminating in increased intracellular 1,25(OH)2D3 and increased production of antimicrobial peptide LL-37, which kills intracellular MTB [6]. Specifically, TLR1/2 activation leads to upregulation of the genes encoding VDR and 1α-hydroxylase (CYP27B1); this allows utilization of 25(OH)D3 and conversion to 1,25(OH)2D3; 1,25(OH)2D3 promotes the formation of the LL-37 antimicrobial peptide from the cathelicidin gene hCAP18, and LL-37 then mediates MTB cell death [6,9,54] (Figure 2). There is now convincing evidence that the antimicrobial peptide LL-37 is an important mediator of this effect [6,9,63], but other downstream mediators also might be recruited, including induction of NOS2A [9,62,64] and phosphatidylinositol-3-kinase (PI3-K)-mediated superoxide production [60].

Successful induction of antimycobacterial activity through cathelicidin and LL-37 production is dependent upon replete serum vitamin D levels [6]. Ethnic groups (including African Americans) at higher risk of TB have lower serum levels of 25(OH)D3 and their serum is less efficient in supporting cathelicidin mRNA induction; supplementation of this serum with 25(OH)D3 restores TLR-mediated induction of cathelicidin mRNA [6]. The findings provide an immunological basis for differential susceptibility among human populations to mycobacterial infection; namely, that vitamin D deficiency portends a higher risk of TB disease.
Inhibition of phagosome maturation is an important example of MTB immune evasion. Killing of phagocytosed organisms is usually achieved through a calcium-dependent fusion process between phagosomes and toxic-acid-containing lysosomes [65]. By inhibiting the fusion step, through lipoarabinomannan-mediated inhibition of sphingosine kinase [65] MTB is able to achieve latency in a protected niche. Recent data demonstrate that MTB-induced phagosome-maturation arrest in a human macrophage model is able to be reversed by vitamin D₃ via a PI3-K pathway [66]. This PI3-K mediation of vitamin D₃ antitubercular activity demonstrates nongenomic (VDR-independent) signaling of vitamin D₃ [60,66]. Thus, both nuclear VDRs, via TLR activation, and non-nuclear receptors, via PI3-K, mediate the effects of vitamin D₃ in the macrophage. Although TLR-mediated MTB killing is an important first line defense against initial TB infection, phagolysosome fusion could play a greater role later in the course of infection, including in the prevention of latent TB reactivation.

Interactions between L-arginine–NO and vitamin D–cathelicidin pathways
Macrophones can be activated either by TLR triggering, leading to increased intracellular vitamin D₃ and cathelicidin induction, or by IFN-γ, acting via Janus tyrosine kinase (JAK) and signal transducer and activator of the transcription (STAT-1)-signaling pathway to upregulate NOS2 and thereby increase NO release (Figure 2). Interactions between the L-arginine–NO and vitamin D–TLR–cathelicidin pathways are probable. First, CYP27b1 requires an extracellular source of L-arginine for full expression and is upregulated by NO in an avian macrophage cell line [28]. Second, the antitubercular effect of vitamin D might be mediated in part by NO [9,62]. Third, nuclear factor kappa B (NF-kB), a downstream mediator of TLR signaling, also might impact on NOS2 regulation and NO production in murine macrophages [67].

These in vitro studies cannot determine how these immunological strategies manifest in the infinitely complex in vivo setting of human TB infection in which there are individual variations in vitamin D and L-arginine concentrations both systemically and locally in the vicinity of the granuloma. Comparative clinical trials of both arginine and/or vitamin D as adjunctive therapies in TB could assess the relative importance of these two immune pathways in the human host.

Potential for attenuation of lung pathology
TB results in significant tissue destruction and long-term impairment of lung function [68]. Attenuation of lung pathology has the potential to minimize long-term pulmonary disability. Both NO and vitamin D share a propensity to bolster innate immune responses but attenuate overexuberant CMI responses [54,55,69,70]. Profound T-cell depression, exemplified by HIV, is significantly detrimental to host containment of mycobacterial growth, but lesser degrees of T-cell depression, such as is achieved with the use of adjunctive corticosteroids [13], are beneficial.

NO inhibits activities including lymphocyte activation and adherence and transmigration of monocytes that contribute to granuloma formation, caseation and lung destruction in TB [69,71]. In TB-infected NOS2− knockout mice and mice treated with NOS2 inhibitors, larger and more destructive lung lesions are seen in early infection even before significant increases in mycobacterial numbers are seen [41,69,72,73]. NO also inhibits production of TNF-α(69), a mediator of caseating necrosis of lung tissue and weight loss in TB. Alveolar macrophage NO production is significantly higher in patients with milder disease than those with more extensive TB, consistent with a disease-protective role for NO not only in mice but also in humans [74].

Although direct evidence for vitamin D ameliorating TB-induced pathology is lacking, indirect evidence would support this hypothesis. The CMI-depressive effect of vitamin D has generated interest in vitamin D₃ as an immunosuppressant treatment after organ transplantation [55]. It also provides the theoretical basis for apparent associations between vitamin D deficiency and autoimmune disease [75]. The possibility of a deleterious effect of replete or supplemented vitamin D stores on immune responses to TB does not appear to be borne out by the literature to date, suggesting that possible harm from Th1 suppression is outweighed by the beneficial roles of vitamin D₃ on macrophage function and in the possible mitigation of tissue damage by suppression of excess Th1 responses.

Clinical experience with L-arginine and vitamin D

Trials of L-arginine and NO in human TB
Extensive experience with therapeutic L-arginine in humans has been gained from clinical trials of L-arginine in the oral, intravenous or inhaled form in studies of both endovascular and immunological functions [53,76–78]. Pharmacokinetic and modest side-effect properties are well described elsewhere [79]. There has been one randomized controlled trial (RCT) of L-arginine in TB to date. Low dose L-arginine supplementation (1 g orally daily) in Ethiopian TB patients was safe and well tolerated [80]. In subgroup analysis of those without HIV infection, there was a modest but significant rise in plasma levels of L-arginine, a significant improvement in the resolution of cough but no significant effect on weight gain and sputum smear conversion in the arginine group. Although arginine was safe and promising, limitations of this study include the small sample size, low dose and apparent posthoc analysis.

Inhaled NO has been trialled as a therapeutic adjuvant in HIV-negative, smear-positive pulmonary TB patients. Administered at a dose of 80 ppm for 72 hr, NO was found to be safe and well tolerated, but no beneficial effects were demonstrated in eight cases compared with ten controls [81]. Definitive conclusions from this study are limited by the small size and short treatment duration.

Measurement of serum NO metabolites can be used to measure systemic, but not organ-specific, NO production,
but adherence to fasting protocols is required [82]. Measurement of exhaled NO (eNO) has been validated as a reliable measure of pulmonary NO production and can now be performed by using portable and simple apparatuses [83,84]. eNO is elevated in human pulmonary TB, decreases with treatment and appears inversely associated with disease severity [70]. Furthermore, NOS2 expression in alveolar macrophages derived from bronchoalveolar lavage correlates significantly with eNO, supporting the validity of eNO in this setting [70]. Finally, systemic administration of L-arginine orally or intravenously increases eNO [76], making eNO a useful means of monitoring response to adjunctive L-arginine.

**Vitamin D trials in TB**

Martineau and colleagues [12] reviewed three RCT and ten prospective case series in which vitamin D was administered to patients with TB, but, overall, the impact of vitamin D supplementation on TB outcome could not be assessed; most studies were conducted in the 1950s, were identified as being of poor quality and used ergocalciferol (vitamin D3), which is less efficacious than cholecalciferol (vitamin D3) [85]. A more recent trial of vitamin D supplementation in Indonesian pulmonary TB patients suggested more rapid sputum clearance and radiological improvement, but the trial was small, the process of randomisation was not described and the safety profile including calcium levels was not assessed [86].

Some [36], but not all [35], epidemiological studies suggest that a fall in serum vitamin D leads to activation of latent TB. If this is the case then an important potential application for vitamin D is as an adjunctive therapy in latently infected individuals to prevent TB reactivation. Clearly, any such clinical trials would need to be very large [87]. However, a recent trial of vitamin D supplementation in latently infected TB patients demonstrated immunological improvement, as indicated by growth restriction of luminescent recombinant Bacillus Calmette-Guerin (BCG)-lux in whole blood from vitamin-D-supplemented people compared with controls [88].

Would vitamin D supplementation be of value only in those who are deficient or could supraphysiological vitamin D levels also be beneficial in those replete at baseline? Ongoing debate regarding ideal vitamin D levels complicates this question [89]; given current recommended reference ranges, vitamin D deficiency is increasingly recognized, even in geographical regions considered to have high UV ratings [90]. It could be hypothesized that because unregulated production of CYP27b1 by activated macrophages (achieving supraphysiological vitamin D levels in some patients) has been naturally selected as an antimycobacterial innate immune response, then there might indeed be an immunological benefit of higher than normal vitamin D concentration in the direct vicinity of the macrophage. The possibility of hypercalcemia would necessitate careful vitamin D dose selection and monitoring and indeed could limit the potential of vitamin D as an adjunctive therapy. The use of vitamin D3 analogues or combination therapy with bisphosphonates has been proposed to exploit the immunomodulatory properties of vitamin D while avoiding possible hypercalcemia and increased bone turnover [55]. However, expensive or complicated regimens, if shown to be of benefit, are unlikely to be widely adopted, especially in resource-poor, high-prevalence TB settings.

**Concluding remarks and further directions**

TB is a disease of the highest global public health significance, requiring the pursuit of innovative research directions (Box 2). Improvements in TB therapeutic regimens through the use of adjunctive immunotherapies could have profound impacts, and these are recognized as priority TB research fields [16]. Evidence for the suitability of L-arginine and vitamin D as candidate adjunctive TB or latent TB treatments include the following: (i) human macrophages use activated vitamin D and L-arginine-derived NO to kill TB bacilli, (ii) human T cells require replete L-arginine levels to function optimally because expression of the CD3ζ chain of the TCR requires L-arginine, (iii) vitamin D and NO might both have the potential to attenuate excessive organ pathology resulting from exaggerated CMI and (iv) active TB infection is associated with deficiencies in L-arginine and vitamin D. The L-arginine and vitamin D immunological pathways might work in parallel in TB-infected individuals, could have differential importance during stages of TB infection or among individuals depending on prevailing genetic, cytokine and substrate influences and are likely to be of varying importance among different host species affected by mycobacteria. Comparative clinical trials of L-arginine and/or vitamin D could help to determine their relative importance in human TB. If L-arginine and/or vitamin D can shorten time to sputum clearance and culture negativity, administration of these agents would have potential for both population and individual benefits, including reduced transmission and possible shorter and, therefore, more successful courses of therapy. Furthermore, with the increasing prevalence of multidrug-resistant TB, agents that improve innate mycobacterial killing mechanisms might improve cure rates in drug-resistant disease. There is, thus, a compelling rationale for clinical trials investigating the safety and efficacy of both L-arginine and vitamin D, singly or together, as adjunctive immunotherapies in TB.

**Acknowledgements**

We acknowledge the many authors who have contributed to the fields reviewed here whose work we have been unable to cite due to space constraints. In keeping with the journal’s requirements, we frequently have been obliged to cite reviews instead. The National Health and Medical Research Council (Australia) supports A.R. (Postgraduate Medical Research scholarship), P.K. (Career Development Award) and N.A. (Practitioner Fellowship, ICRG and Program Grant).

**References**

76 Yes, T.W. et al. (2007) Impaired nitric oxide bioavailability and L-arginine reversible endothelial dysfunction in adults with falciaparum malaria. J. Exp. Med. 204, 2693–2704
**Figure 1.** Metabolic fate of L-arginine and macrophage nitric oxide production. Sources of L-arginine (the active biological enantiomer of this conditionally essential amino acid) in the fed state include exogenous (food) and endogenous sources (whole-body protein degradation and, to a small extent, de novo synthesis from citrulline by renal arginosuccinate synthase) [22]. L-arginine is converted to NO by NOS2, the inducible product of the NOS2 gene. Th1 cytokines (principally IFN-γ) stimulate expression of macrophage NOS2 leading to NO production, whereas under the influence of Th2 cytokines (IL-4, IL-10 and IL-13), arginine is depleted by arginases. NO is one of several RNIs with antimicrobial activity [91].

**Figure 2.** Simplified schemata of macrophage antmycobacterial pathways. Parallel pathways [IFN-γ–JAK and STAT–L-arginine-derived NO] and [TLR2–MyD88 and NFκB–vitamin D–cathelicidin and LL37] lead to macrophage activation and mycobacterial control. IFN-γ release by Th1 and NK cells (stimulated by MTB-infected antigen-presenting cells) engages the JAK and STAT-1 pathways within the macrophage, leading to upregulation of genes including NOS2. Interaction of MTB with TLR in monocytes and macrophages (mainly via TLR1/2) leads to engagement with MyD88 and NFκB. The subsequent cascade of antmycobacterial responses includes TNFα production, IL-12 release (recruiting Th1-mediated immunity, providing another means of IFN-γ production and further macrophage activation) and upregulation of the vitamin D3–cathelicidin pathway. High NO concentrations (e.g. resulting from high L-arginine concentration and NOS2 expression) are associated with MTB growth inhibition, whereas low-level NO (e.g. resulting from L-arginine or NOS2 insufficiency) appears to be associated with MTB dormancy. Abbreviations: IFN-γ, Interferon gamma; JAK, Janus tyrosine kinase; MTB, Mycobacterium tuberculosis; MyD88, adaptor protein; NFκB, nuclear factor-kappa B (cytoplasmic transcription factor); NK cell, natural killer cell; STAT, signal transducer and activator of transcription; TLR2, Toll-like receptor 2; Th1, Type 1 T helper cell; VDR, vitamin D receptor. Dotted arrow signifies non-dominant pathway.

**Box 1.** Chronology of vitamin D-based treatments in the pre-antibiotic era

<table>
<thead>
<tr>
<th>Year</th>
<th>Event</th>
</tr>
</thead>
<tbody>
<tr>
<td>1855</td>
<td>Rikli, a Swiss doctor, opened a thermal treatment station in Slovenia.</td>
</tr>
<tr>
<td>1901</td>
<td>Finsen was awarded the Nobel Prize for Medicine for successful treatment of cutaneous tuberculosis (lupus vulgaris) with UV radiation.</td>
</tr>
<tr>
<td>1903</td>
<td>Rollier from Switzerland opened a hospital to treat TB by using graded sun exposure. In 1914 he published a book based on results.</td>
</tr>
<tr>
<td>1922</td>
<td>Increasing popularity of sunlight treatment in the UK. Establishment in the UK of ‘Committee on Sunlight’ and Light Department at London Hospital; widespread adoption of sun-exposure practices.</td>
</tr>
<tr>
<td>1771</td>
<td>Cod liver oil was introduced into the British pharmacopoeia.</td>
</tr>
<tr>
<td>1833</td>
<td>Heinke, a German doctor, reported on the successful use of cod liver oil in the treatment of TB.</td>
</tr>
<tr>
<td>1841</td>
<td>Bennett, a Scottish physician, published medical uses of cod liver oil, including in TB. Five case reports of improvement in TB symptoms, but two relapsed after stopping cod liver oil.</td>
</tr>
<tr>
<td>1849</td>
<td>Hospital for Consumption and Diseases of the Chest in London published outcomes of cod liver oil in TB: 18% disease arrested, 63% improved and 19% unchanged.</td>
</tr>
<tr>
<td>1855</td>
<td>Woods from Philadelphia, USA, attributed the 19% fall in deaths due to TB between 1847 and 1852 to the use of cod liver oil.</td>
</tr>
</tbody>
</table>

**Box 2.** Future research directions
Adjunctive immunotherapies, new TB drugs, new regimens of existing drugs and novel means of drug delivery (e.g. liposomal rifabutin and oral streptomycin) are among the strategies being investigated to improve treatment regimens for both sensitive and multidrug-resistant TB.


Pending results of such trials, nutrition needs to be optimized in people with TB and vitamin D deficiency should be identified and corrected if present.

Table 1. Immunological evidence for a role of arginine and NO in TB control

<table>
<thead>
<tr>
<th>Study Type</th>
<th>Results</th>
<th>Refs</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>L-arginine-derived nitric oxide and other RNIs</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>In vitro MTB culture</em></td>
<td>MTB isolates are sensitive to NO and other RNI and differ in their RNI-susceptibility.</td>
<td>[50]</td>
</tr>
<tr>
<td><em>Mouse macrophage studies.</em></td>
<td>Arginine-derived RNI in mouse macrophages effectively kill MTB.</td>
<td>[41]</td>
</tr>
<tr>
<td><em>Human macrophage studies.</em></td>
<td>MTB lacking genetic resistance to RNI cannot grow in mouse macrophages, in contrast with wild-type MTB.</td>
<td>[42]</td>
</tr>
<tr>
<td><em>Blood mononuclear cells from healthy donors infected ex vivo with MTB and from people with pre-existing TB infection produce NO.</em></td>
<td>Alveolar macrophages from healthy humans infected ex vivo with MTB produce NO, and NO production correlates with intracellular growth inhibition of MTB.</td>
<td>[45]</td>
</tr>
<tr>
<td><em>Blood mononuclear cells from healthy donors infected ex vivo with MTB and from people with pre-existing TB infection produce NO.</em></td>
<td>Blood mononuclear cells from healthy donors infected ex vivo with MTB and from people with pre-existing TB infection produce NO.</td>
<td>[92]</td>
</tr>
<tr>
<td><em>Human macrophage studies.</em></td>
<td>Pulmonary macrophages kill mycobacteria only if they express NOS2; killing is prevented with a NOS inhibitor.</td>
<td>[93]</td>
</tr>
<tr>
<td><em>NOS2 expression is increased in peripheral blood monocytes from people with TB compared with healthy controls. NOS2 is expressed in macrophages from lungs of patients with tuberculosis.</em></td>
<td>NOS2 expression is increased in peripheral blood monocytes from people with TB compared with healthy controls. NOS2 is expressed in macrophages from lungs of patients with tuberculosis.</td>
<td>[70]</td>
</tr>
<tr>
<td><em>NOS2 expression is increased in peripheral blood monocytes from people with TB compared with healthy controls.</em></td>
<td>Pulmonary NO production measured by eNO is significantly higher in TB patients than in controls; eNO is inversely associated with disease severity and decreases with anti-TB treatment.</td>
<td>[95]</td>
</tr>
<tr>
<td><em>Peripheral blood T cells from patients with pulmonary TB have decreased CD3ζ expression, correlating with arginase-mediated L-arginine deficiency, which normalises with successful TB treatment.</em></td>
<td>Pulmonary NO production measured by eNO is significantly higher in TB patients than in controls; eNO is inversely associated with disease severity and decreases with anti-TB treatment.</td>
<td>[70]</td>
</tr>
</tbody>
</table>

**Relationship between CD3ζ expression and L-arginine in TB and other conditions**

| Human T cells in vitro | Depletion of L-arginine in vitro causes reduced CD3ζ expression, impaired T-cell signaling and diminished proliferation. | [91] |
| Arginase-mediated L-arginine depletion induces downregulation of CD3ζ; addition of L-arginine ex vivo leads to CD3ζ re-expression and recovery of T-cell proliferation. | [26] |
| Peripheral blood T cells from patients with pulmonary TB have decreased CD3ζ expression, correlating with arginase-mediated L-arginine deficiency, which normalises with successful TB treatment. | [23] |
LITERATURE UPDATE

Vitamin D in TB

Since publication of the above paper, results of a randomised trial of supplemental cholecalciferol (100 000 IU) in patients with TB in Guinea-Bissau have been reported, demonstrating no mortality difference between vitamin D supplement and placebo arms.81 Due to funding constraints, the study was terminated after only 63% of the planned sample size was enrolled; furthermore, only 64% of those enrolled were able to contribute primary outcome data (a TB score generated by the authors). The study did include an assessment of smear positivity at 1 month (in 58% of participants) and there was a non-significant reduction in smear positivity in the vitamin D group (risk difference -6%, 95% CI -18 to 5). Respiratory function was not assessed. Interestingly, the study did not demonstrate any difference in vitamin D levels between the 2 groups, suggesting that the vitamin D formulation used (an injectable preparation given orally) may have been poorly absorbed. Finally, while the study was randomised, the use of treatment group medication labels (‘lot 204’ or ‘lot 205’) meant that allocation concealment and blinding could not be guaranteed.81 Therefore this study is inconclusive and indicates the need for further adequately powered trials of vitamin D in TB.

This publication and its accompanying editorial82 fuelled ongoing debate regarding the appropriate timing of administration of vitamin D in relation to onset of active TB. While reliable, reproducible clinical trial results in both latent or active TB are still lacking, some hypothesise that vitamin D supplementation would only be efficacious as a prophylactic agent for reducing the risk of activation of latent TB (for instance, as lifelong therapy in vitamin D-deficient people with latent TB).83 Others argue that vitamin D has roles in immunological pathways other than just latent TB reactivation, thereby warranting trials of vitamin D as an adjunctive agent in active TB.84

In preliminary safety data from another study, 11 TB patients were administered a single dose of 100 000 IU ergocalciferol (vitamin D2, considered less efficacious than D385), resulting in a significant rise in serum vitamin D2 and no episodes of hypercalcaemia at 8 weeks; no rise in serum D2 occurred in 14 TB patients randomised to placebo.86 Although the most common time for hypercalcaemia occurrence has been
reported to be around 2 to 4 weeks\(^8\),\(^8\) these authors only examined hypercalcaemia at 8 weeks, therefore potentially underestimating true hypercalcaemia occurrence.

Contrasting with the majority of reports showing that low plasma vitamin D correlates with active TB, Selvaraj et al recently reported that plasma 1,25(OH)\(_2\)D\(_3\) levels were significantly increased among pulmonary TB patients compared to healthy controls.\(^8\) Only 1,25(OH)\(_2\)D\(_3\) (not 25(OH)D\(_3\)) was measured in this study. The authors hypothesised that the widely reported finding of low 25(OH)D\(_3\) in people with active TB\(^9\) may be explained by excessive conversion of 25(OH)D\(_3\) to 1,25(OH)\(_2\)D\(_3\), providing a mechanism for simultaneous findings of low 25(OH)D\(_3\), and 1\(\alpha\)-hydroxylase-driven hypercalcaemia which can characterise active TB.\(^8\)

A further update since publication of our paper\(^3\) pertains to VDR polymorphisms: subsequent to Lewis et al’s meta-analysis of VDR polymorphisms\(^9\) which we cited, Gao and colleagues have now reviewed the literature, and report that among Asians, the FokI ff VDR genotype is associated with increased TB risk, the BsmI bb genotype was protective, and TaqI and ApaI polymorphisms were only marginally significant.\(^9\)

**Nitric oxide in TB**

A subsequent review of NO in mycobacterial infection, referencing our article, offered similar conclusions regarding the role of L-arginine-derived NO in TB.\(^9\) Also furthering the understanding of mechanisms of immune evasion and intracellular survival, El Kasmi and colleagues reported that arginase can be induced by MTB in macrophages, thus diverting L-arginine substrate away from NO production and instead promoting the breakdown of L-arginine to ornithine.\(^9\) This might serve as a microorganism-mediated mechanism to aid the establishment of successful latent infection.

Of particular relevance, a recent study found that 1,25(OH)\(_2\)D\(_3\), in combination with IFN\(\gamma\), led to the up-regulation of NOS2 mRNA and NO production by human peripheral blood monocytes / macrophages after stimulation with MTB or PPD, and that this response was attenuated by selective TKR2 antibodies.\(^9\) These data indicate for the first time that NO production may comprise part of the anti-mycobacterial mechanism of action of vitamin D\(_3\), via a TLR2 mechanism.
In a mouse study, Beisiegel et al\textsuperscript{96} have provided new evidence of a lung protective role of NO in the murine immune response to infection with virulent MTB, whereby NO is required for regulation of pulmonary neutrophil influx. When wild type and NOS2 (iNOS) knockout mice were infected with virulent (H37Rv) and attenuated (H37Ra) MTB strains, iNOS knockout mice showed grossly impaired responses, including abnormal neutrophil influx, when infected with the virulent strain.

These updates reinforce the arguments put forward in the review article\textsuperscript{38} of the importance of undertaking investigation of adjunctive therapies such as L-arginine / NO and vitamin D in TB.
4 Introduction III: Nitric oxide and its measurement in vivo

“A startlingly simple molecule unites neuroscience, physiology, and immunology, and revises scientists’ understanding of how cells communicate and defend themselves.”

The discovery of NO as not just a noxious environmental pollutant, but an important biological mediator, was made in the late 1980s, revealing this as the agent previously known as endothelium-derived relaxing factor. Analogous with the current cross-disciplinary interest in vitamin D and its new-found pleiotrophic actions, NO was found to have potent roles in cell signalling in many organs, earning it the title of ‘molecule of the year’ in 1992, and opening the door to enthusiastic interest in the therapeutic potential of NO and its precursor, L-arginine, and to ways of measuring NO in vivo.

An understanding of the antimycobacterial function of NO, summarised above, underpins the use of L-arginine in the AVDAPT clinical trial. A key measure of interest in AVDAPT study participants is fractional exhaled nitric oxide (FE\textsubscript{NO}). This chapter therefore reviews the literature pertaining to FE\textsubscript{NO} measurement.

4.1 NITRIC OXIDE PATHWAYS

NO is derived from L-arginine by nitric oxide synthase (NOS) enzymes, including the 2 constitutive NOS enzymes (neuronal NOS [nNOS or NOS1] and endothelial NOS [eNOS or NOS3], and inducible NOS (iNOS or NOS2). NOS2 is the NOS enzyme subtype of chief interest in the lung and in immunity. Induction of NOS2 gene expression by pro-inflammatory cytokines has been summarised above. This gene is expressed widely in different cell lines in the respiratory tract (alveolar macrophages, airway and vascular smooth muscle cells, epithelial and endothelial cells, lung fibroblasts, mast cells and neutrophils), consistent with the wide actions of NO. Biologically beneficial or protective effects of NO in the lung (e.g. smooth muscle relaxation, downregulation of Th1 pro-inflammatory activities, and the killing of
invading microorganisms) are balanced by the potentially deleterious effects of NO and other reactive nitrogen species (e.g. pro-inflammatory activities, necrosis, apoptosis).  

NO is a free radical (it has one unpaired electron) making it highly reactive with a short half life in vivo of 1 to 5 seconds. This short half life precludes direct NO measurement in blood. Surrogate measures such as NO metabolic products (e.g. nitrite, NO2−), or urinary excretion rates of nitrate (NO3−), have been used in the past. Measurement of FE.NO provides a means of directly determining NO production in a human organ system. Therefore this method has potential utility in examining NO dynamics in lower respiratory tract infections such as TB.

4.2 EXHALED NITRIC OXIDE MEASUREMENT

FE.NO can be used as a marker of airway inflammation. A joint position statement of the European Respiratory and American Thoracic Societies provides guidelines for FE.NO measurement.

METHODS

The current standard method for FE.NO measurement is a single-breath technique comprising inhalation to total lung capacity followed by exhalation at a constant flow rate of 50 ± 5 mL/s (achieved by real-time audio and visual feedback from the measuring device) for a 10s duration. Modern portable devices permit online measurement (i.e. providing a real-time display of FE.NO). Alternatively, offline testing techniques can be employed where exhalate is collected into an impermeable bag for delayed analysis. The principles of FE.NO measurement are listed in Box 4.1.

Devices available for FE.NO measurement include large chemiluminescence analyzers such as the NiOX FLEX® (Aerocine, Sweden), and hand-held portable machines such as the NiOX MINO® (Aerocine, Sweden) (See Figures 4.1 and 4.2). Constraints of using traditional non-portable FE.NO analysers in a tropical clinic setting include the need for a large research space and air conditioning. The advent of portable eNO analysers in part overcomes these barriers. The hand-held portable NiOX MINO® has been reported to provide valid, reproducible measures of FE.NO.
Box 4.1: Principles of exhaled nitric oxide measurement

- Inhalation of NO-free air
  - Can be achieved by use of an NO scrubber in the mouthpiece which absorbs NO from inspired air.

- Expiration at a fixed flow rate:
  - $F_{\text{ENO}}$ varies markedly with flow rate (rises non-linearly with increasing flow rate).
  - The standard current flow rate required for most analysers, considered to be reproducibly achievable by adults and children, is 50 mL/s.

- Avoidance of nasal NO
  - Nasal NO is high relative to NO in lower airways.
  - High pressures created in the oral cavity during exhalation against resistance ensures velum closure; this is generally considered a satisfactory technique to exclude nasal air, avoiding the need for a nose peg.

- Avoidance of breath-holding
  - Breath-holding results in accumulation of NO, therefore the inspiration should be immediately followed by expiration.

- Avoidance of NO or arginine-containing foods
  - Foods rich in nitrates or nitrites (e.g. lettuce) or arginine (e.g. peanuts) should be avoided prior to testing; thus fasting for 2 hours prior to measurement is recommended.

- Appreciation of the impact of smoking
  - Smoking acutely decreases $eNO$.

- Avoidance of exercising prior to $eNO$ measurement
  - Exercise has been reported to have variable effects on $eNO$, therefore rest for 1 hour is recommended prior to $eNO$ testing.

Box 4.1 references: \textsuperscript{100, 104}

**NORMAL $F_{\text{ENO}}$ RANGE**

The understanding of ‘normal’ $F_{\text{ENO}}$ and its determinants is evolving. Upper limits of normal $F_{\text{ENO}}$ at a flow rate of 50mL/s ($F_{\text{ENO0.05}}$) for healthy adults and children of 33 ppb and 25 ppb, respectively, have been suggested.\textsuperscript{105} $F_{\text{ENO0.05}}$ distribution shows a right skew; median $F_{\text{ENO}}$ in healthy Swedish non-smoking subjects was found to be 10 ppb (range ~5 to 55),\textsuperscript{106} and very similar findings (median $F_{\text{ENO}}$ 16.5 ppb, range 4.9 – 67.1) were reported from healthy Papuan and Non-Papuan adults by Yeo and colleagues at the MSHR-NIHRD Timika field research site.\textsuperscript{56}
Positive associations were reported between $\text{FE}_{\text{NO}}$ and height and age, but not sex, in a carefully conducted survey of $\text{FE}_{\text{NO}}$ values in 1131 healthy, never-smoking Swedish adults.\(^{107}\) The upper limit of normal $\text{FE}_{\text{NO}}$ for 25-34 year olds $<$160cm was reported as 24.0 ppb, and for 65-75 year olds $>$190 cm, as 57.4 ppb.\(^{107}\) However even excluding asthma, other lung diseases, atopy and smoking, the authors of this study found $\text{FE}_{\text{NO}}$ to be poorly predictable based on the explanatory variables height and age, suggesting that other factors including nutritional status, genetics and / or undiagnosed or low grade lung inflammation might contribute to inter-subject $\text{FE}_{\text{NO}}$ variability.

**Figure 4.1: NiOX FLEX**

*Study investigator (Anna Ralph) demonstrating use of the NiOX FLEX. Photo: Daniel Lampah.*

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**CLINICAL UTILITY**

Use of $\text{FE}_{\text{NO}}$ as a monitoring tool is best described in asthma. Increases herald an asthma exacerbation, and $\text{FE}_{\text{NO}}$ evaluation has been suggested as a means of diagnosing asthma, monitoring response to treatment, verifying adherence, and predicting upcoming exacerbations.\(^{100,108}\)
Figure 4.2: NiOX MINO
Research assistant (Govert Waramori) instructing a healthy volunteer in the use of the portable NiOX MINO. Photo: Anna Ralph.

Although an understanding of what constitutes normal $FE_{NO}$ remains incompletely defined, Table 4.1 illustrates selected states found to be associated with abnormal $FE_{NO}$. Some factors have been associated with both increases and decreases in $FE_{NO}$ (exercise, caffeine, sputum induction).\textsuperscript{109}

Table 4.1: Factors associated with abnormal exhaled nitric oxide

<table>
<thead>
<tr>
<th>Factors associated with increased exhaled nitric oxide</th>
<th>Factors associated with decreased exhaled nitric oxide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asthma exacerbation</td>
<td>Cystic fibrosis (chronically)</td>
</tr>
<tr>
<td>Airway viral infection</td>
<td>Active and passive smoking</td>
</tr>
<tr>
<td>Alveolitis</td>
<td>Alcohol</td>
</tr>
<tr>
<td>Asbestosis</td>
<td>Altitude</td>
</tr>
<tr>
<td>Bronchiectasis</td>
<td>Severe malaria</td>
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<tr>
<td>Chronic bronchitis</td>
<td></td>
</tr>
<tr>
<td>Lung transplant rejection</td>
<td></td>
</tr>
<tr>
<td>Cystic fibrosis (during acute exacerbation)</td>
<td></td>
</tr>
<tr>
<td>Increasing age and height</td>
<td></td>
</tr>
<tr>
<td>Height</td>
<td></td>
</tr>
<tr>
<td>Nitrate-rich diet</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1 references\textsuperscript{56, 109}
EXHALED NITRIC OXIDE IN TB

Two research groups have investigated \( \text{FeNO} \) in pulmonary TB and reported inconsistent findings. Wang and colleagues in Taiwan\(^{110} \) conducted a detailed study (findings from which are included in our paper above\(^{38} \)) in a small number of people (19 adults with pulmonary TB and 14 control subjects), in which \( \text{FeNO} \) was correlated with disease stage and severity, and with other markers of NO production (expression of \( \text{NOS2} \) gene in alveolar macrophages obtained via bronchoalveolar lavage, and in lung tissue [TB patients only], obtained via transbronchial biopsy). \( \text{FeNO} \) was measured using a now-obsolete method, whereby patients exhaled slowly (at an uncontrolled flow rate of around 200 mL/s) through an open tube, with a probe sampling from the stream of expired breath. Therefore results in parts per billion are not comparable to results obtained at flow rates of 50 mL/s. They found \( \text{FeNO} \) to be significantly higher in people with newly diagnosed TB compared with controls, but with normalisation of \( \text{FeNO} \) by 3 months of treatment. An inverse association between \( \text{FeNO} \) and disease severity was found, which the authors hypothesised might indicate that people with a poorer capacity to generate NO from alveolar macrophages developed more extensive disease. \( \text{NOS2} \) gene expression in alveolar macrophages derived from bronchoalveolar lavage correlated significantly with exhaled NO, supporting the validity of \( \text{FeNO} \) as a measure of lung NO production in this setting.\(^{110} \)

In contrast, Idh and colleagues a decade later, in a paper which appeared after publication of our review,\(^{38} \) reported inconsistent \( \text{FeNO} \) results in Ethiopian adults with smear positive pulmonary TB.\(^{111} \) Several methodological flaws were evident in their report. \( \text{FeNO} \) was measured using a non-portable NiOX analyser but omitted the standard initial step of inhalation of NO-free air prior to exhalation. Analyses did not control for age or anthropometric measures. Performing separate pair-wise comparisons between 4 patient subgroups: people with TB and HIV (n=36); people with TB without HIV (n=59); household contacts (n=17); blood donors (n=46), the authors reported that mean \( \text{FeNO} \) was lower in HIV-TB co-infected patients (approximately 14 ppb) than in controls (approximately 18 ppb), and TB patients without HIV were more likely to have \( \text{FeNO} > 25 \) ppb (an arbitrary post-hoc cut-off) than TB patients with HIV. No correlation was found between \( \text{FeNO} \) and radiological disease severity. While only limited conclusions can be drawn from this study, results do appear to support the hypothesis that a poorer capacity to generate NO, as seen in the HIV positive group of TB patients, might be
consistent with these people having a defective immune response, while the immunocompetent group with TB were more likely to have elevated eNO readings, consistent with NOS2 upregulation as part of the antimycobacterial immune response.

The conflicting results from these studies may partly reflect change in FeNO measurement methodology over time, particularly flow rate. They indicate the requirement for further investigation of FeNO in people with pulmonary TB, to clarify questions of pathophysiology, addressed further in Chapter 11.
5 Study setting

5.1 TIMIKA FIELD RESEARCH FACILITY

The Timika Translational Research Facility in Timika, Mimika district, Papua Province, Republic of Indonesia, is operated by Menzies School of Health Research’s (MSHR) International Health Division in partnership with the National Institute for Health Research and Development (NIHRD), Indonesian Ministry of Health. Since establishment of this facility in 1999, major research into malarial drug resistance and comparative drug studies in *Plasmodium falciparum* and *P. vivax* infections, have led to important policy changes in local malaria management. Previous MSHR-NIHRD tuberculosis research at the site in 2003-4 has documented MTB drug resistance rates, sputum transportation methods, TB-induced lung impairment, and described the model of TB care delivery in Timika.

Figure 5.1: Map of Indonesia and Northern Australia showing Timika (Papua Province)

Additional to the TB research currently underway, malaria research at the field site includes ongoing surveillance of the impact of community-based artemisinin combination drug deployment on uncomplicated and severe falciparum and vivax malaria, impact on pregnancy outcomes, in vitro drug sensitivity testing for *P. falciparum* and *P. vivax*, and a phase 2 study of intravenous arginine as adjunctive
therapy in patients with severe malaria. Studies are also being conducted in sepsis and Maternal and Child health (see http://www.menzies.edu.au/research/international-health). The Research Facility currently receives core infrastructure financial support from AusAID, and a National Health and Medical Research Council (Australia) grant was provided in 2009 for ongoing TB research.

Figure 5.2: Timika community hospital (Rumah Sakit Mitra Masyarakat)
*Photos: Anna Ralph*

(a) Hospital entrance

(b) Hospital sign and Papuan carvings

(c) Nicholas Anstey, Enny Kenangalem & Paul Kelly at RSMM
Strong collaborations are in place with important local health organisations, including the District Health Authority, the two local hospitals, the Amungme and Kamoro Community Development Institution, and the Public Health Malaria Control unit of Freeport Indonesia. The field research facility, comprising a laboratory, offices and accommodation for visiting research staff is located on the campus of the community hospital, Rumah Sakit Mitra Masyarakat (RSMM) (Figure 5.2), 15 km from the Timika town centre and TB clinic.

Figure 5.3: NIHRD-MSHR Timika research staff and visitors in the research building
L to R: Back row: Gysje Pontororing, Meryani Girsang (NIHRD), Wendelina Fobia, Yani Reyaan, Natalia Dwi Haryanti
Front row: Anna Ralph, Trivonita Ria Bless, Getruida Bellatrix, Denius (visitor)
Photo: Daniel Lampah.

5.2 TIMIKA TB CLINIC

The Timika TB clinic (Figure 5.4) is situated at the Timika Community Health Centre (Pusat Kesehatan Masyarakat, abbreviated Puskesmas), run by the District Government Health Authority (Dinas Kesehatan). As well as the TB clinic, the Puskesmas campus includes a general clinic, two sexually transmitted infection (STI) clinics and a laboratory. The general clinic and one STI clinic are operated by Dinas Kesehatan, while the TB clinic and one STI clinic are funded by the Public Health Malaria Control unit of PT Freeport Indonesia, and the laboratory by both sources. The TB clinic is the main referral centre for outpatient management of TB in Timika. Patients requiring
hospitalization are referred to either of the two Timika hospitals. Although some smaller Puskesmas clinics also provide TB management, they are regularly encouraged by the research staff to refer TB patients for management at the dedicated Timika TB clinic, in keeping with District Health Authority policy, since treatment success can be optimized there by TB specialist staff, including public health employees who trace non-attendees in the community. Space at the clinic was very limited at the time of study commencement, so an extension to the existing facility was built in 2008 (using Covance Award funding granted to me by the Royal Australasian College of Physicians) (Figure 5.4).

Figure 5.4: Timika TB clinic and staff
Photos: Anna Ralph and Govert Waramori
(a) Front entrance
L to R: Anna Ralph, Daud Rumere (NIHRD-MSHR lab technician), Kristina Palupi (NIHRD).
(b) Research staff
L to R: Gysje Pontororing, Govert Waramori, Getruida Bellatrix.
(c) Before construction of extension
(d) After construction of extension

5.3 OVERVIEW OF TIMIKA

Indonesia is the world’s fourth most populous nation, at 237 million people. Defining characteristics of the Indonesian archipelago are cultural, linguistic and religious diversity. These are especially evident in its easternmost province of Papua, as
photographs of staff and study participants in this thesis attest. Known outside of Indonesia as West Papua, this western half of the island of New Guinea was called Irian Jaya during Sukarno’s presidency, but was re-named Papua Province in 1999.116 The provincial capital is Jayapura on the north coast.

Figure 5.5: Mimika district map

(a) Districts of Papua Province (Mimika region = 12)  Map from ref 123 (IBBS 2006 survey)

(b) Mimika region showing Timika location  Map courtesy of Public Health / Malaria Control, Timika.
The Mimika district on the southern coast of Papua covers an area of 21,522 square-kilometres with 12 sub-districts and 85 villages. Timika, the largest town in Mimika district, is in equatorial lowlands rainforest, 3 degrees south of the equator (Figures 5.1 and 5.5). Climatic conditions are dramatic, with rainfall estimates around Timika and mountain foothills areas between 4 and 11m annually. This quantity of rain and the accompanying storms, floods and power blackouts, impact significantly on day-to-day life. Timika lacks a distinct monsoon season, although rainfall peaks in July to September and again in December. The lack of major seasonal variation in UV exposure removes what might pose a confounder in this vitamin D-related research, making Timika a uniquely ideal environment for the AVDAPT study.

When the rain clouds infrequently clear, the mountain ranges of the Lorenz National Park, and its visible mountaintop glaciation, are revealed to be unexpectedly close to Timika. The dominant peak Puncak Jaya (Cartenz Pyramid) reaches 4883 m, making it the highest mountain in Australasia / Oceania / South-East Asia region (see http://www.papuaweb.org/). The area is thought to have one of the highest rates of biodiversity in South-east Asia.

**HISTORICAL AND SOCIAL BACKGROUND**

“Papua’s rapid transformation from being one of the world’s most isolated societies to becoming part, initially, of a large multi-ethnic colonial empire (Dutch East Indies), then an independent state (Indonesia), together with the disjunction between the colonial era international boundaries and the complex messy intermingling of Malay and Melanesian cultural worlds, have framed the conflicts of the past century.”

Indonesian independence from the Netherlands was declared by the first Indonesian president, Sukarno, in August 1945. Papua was however the subject of an ongoing dispute between Indonesia and the Netherlands, until the Dutch administration ultimately departed in 1962 and the territory was transferred to Indonesia via the interim authority of the United Nations. An “Act of Free Choice” led to Papua’s incorporation into Indonesia and the subsequent development of the Free Papua movement, setting the scene for Papua’s contemporary socio-political climate, which continues to attract adjectives such as ‘restive’ or ‘politically sensitive’.

The last 30 years in Timika have been characterised by immense cultural shifts due to the discovery of mineral wealth in the Grasberg Mountain range. Mining of Ertzberg commenced in the 1960s, and expanded to the nearby highly lucrative Grasberg deposit.
in 1988. The mine is now one of the largest global producers of copper and gold, operated by PT Freeport Indonesia and 86% owned by American Freeport McMoRan Copper and Gold. Mining operations have most particularly impacted the Papuan tribes-people of the seven Mimika district tribes (Amunge, Kamoro, Dani, Damal, Nduga, Moni and Mee). Since 1995, 1% of funds from gross annual revenue from the Freeport-McMoRan mine have been allocated to “community development” for these seven tribes. Use of these funds, managed by the organisation Lembaga Pengembangan Masyarakat Amungme dan Kamoro (LPMAK) (Amungme and Kamoro Community Development Institution; see http://www.lpmak.org), has included construction and operation of the hospital (RSMM), and construction of the MSHR-NIHRD research facility building.

Shifting population dynamics resulting from mining activities and government-sponsored transmigrants have resulted in growth in Timika’s population from ~3000 in 1967 to 130 000 in 2004 and an estimated 200 000 in 2009, representing the highest rate of growth of any urban community in Indonesia. The number of Non-Papuans in Timika is now approximately equal to numbers of indigenous Papuans, although a 2006 survey found the estimated population breakdown throughout the whole province to be 69% Papuan / 31% Non-Papuan. This survey also illustrated a bottom-heavy population pyramid (80% aged less than 40; average age 30 years), distributed throughout lowland (70%) and highland (30%) districts, many of which are difficult to access. Farming and fishing were common occupations (43% of survey respondents), and unemployment was high (31%). Close to half of females surveyed (42%) and 29% of males had never attended school.

Many hundreds of Papuan languages are spoken. The official language throughout the country since 1962 is Bahasa Indonesia, a standardised dialect of Malay. Knowledge of Bahasa Indonesia is high among Timika residents, but more limited among Papuans from isolated areas or who have received limited or no schooling. Of the Papuan participants in the AVDAPT study, approximately 10% require a Papuan-Indonesian translator. Identification with a religious belief is considered an integral component of Indonesian identity. Further illustrating aspects of population diversity in Papua Province, Papuans mostly follow various Christian denominations introduced by earlier missionaries, and Non-Papuans identify predominantly as Muslim followed by Protestant, Catholic, Buddhist or Hindu.
HEALTH STATUS AND HEALTH CARE

In addition to the community hospital described above (RSMM), which provides free health care to the Papuans of the seven tribes, a second Regional General hospital, Rumah Sakit Umum Daerah, opened in September 2008, where low fees are charged for health care provision.

Health status and health determinants (education, socio-economic status) are poor in Papua Province compared with Indonesia as a whole. The Mimika district is characterised by malaria endemicity, with multidrug-resistant strains of *P. falciparum* and *P. vivax* being highly prevalent. The first reports of chloroquine-resistant *P. vivax* were from Papua (and Papua New Guinea) in 1989, and these regions now have the highest rates of drug-resistant *P. vivax* in the world. The calculated incidence of malaria is 512 and 322 per 1000 population per year for *P. falciparum* and *P. vivax* respectively.

Maternal mortality and infant mortality rates in Mimika district are exceptionally high, exceeding those reported elsewhere in Indonesia, at 1145 maternal deaths per 100 000 live births, and 68 infant deaths per 1000 live births. Less than 40% of pregnant women access antenatal care visits or deliver in hospital.

The reported HIV prevalence in Papua Province in 2007 was 60.9 per 100 000 population, 15 times the Indonesian national average, and cumulative AIDS cases in Papua reported to March 2007 were second highest in the country after Jakarta. In a 2006 population survey, 52% of people had heard of HIV / AIDS, but the percentage was considerable lower (26%) in those with no or limited education. Only 35% of people surveyed knew that condom use was protective. Public awareness campaigns to redress this are becoming evident (Figure 5.6). HIV / AIDS is deeply stigmatised in Papua. The Jakarta Post reported on November 22nd 2008:

“Amid protests from Papuans and NGOs, the Papua provincial legislative council is set to pass a bylaw on HIV/AIDS that includes a controversial article requiring certain people living with the disease to be implanted with a microchip...the microchips would only be implanted in people living with HIV/AIDS who were deemed to be...‘actively seeking sexual intercourse’.”
Although the microchip plan was subsequently overturned, such approaches to public health illustrate the formidable barriers that exist to the provision of optimal HIV care in Papua.

Figure 5.6: HIV education banner near RSMM hospital
Photo: Anna Ralph

5.4 CONDUCT OF MEDICAL RESEARCH IN PAPUA

Authorisation to conduct medical research in the province is complex and regulated, currently requiring approval from 2 Indonesian government ministries (Ministry of Health and Ministry of Research and Technology). Other required approvals are listed in Box 5.1.

5.5 CONCLUSION

Working in Papua Province, alongside the many local and national research partners to undertake this doctoral research, has offered a unique and highly valued opportunity to stay in an infrequently-visited tropical rainforest region of great geographical and historical interest, and to gain personal insights into a richly complex society.
Box 5.1: Approvals required for conducting medical research in Papua, Indonesia

Ethics committee approvals gained from:
- National Institute of Health Research Development, Indonesia
- Menzies School of Health Research, Darwin
- Australian National University, Canberra

Training in Good Clinical Practice (GCP)
- Required by study investigators (see Appendix 15.2)

Authorisation to conduct research and receive multi-entry permit
- The Study protocol, curricula vitae and health certificates of study investigators, forms of identification and letters of support from the Indonesian Ministry of Health, were submitted to the Ministry of Research and Technology, Indonesia. Permission to conduct research and a letter of support to Police headquarters were subsequently secured 3 months later from the Ministry of Research and Technology.
- After registration with Police Headquarters in Jakarta and Papua, a letter of introduction facilitating research in Papua province was issued.

Permission from Badam POM (National Food and Drug Administration, Indonesia) to import medications
- Prior to study commencement, Badam POM required details of study medications (ingredients, manufacturer, packaging, batch numbers, expiry dates, numbers of tablets to be imported, whether manufactured according to Good Manufacturing Practice standards and Halal requirements) and rationale for medication importation.
- Permission to import medications granted June 2008.

Permission to perform laboratory tests at designated reference laboratories in Australia
- Materials Transfer Agreements were signed as described in Chapter 6 (Box 6.1)

Figure 5.7: Images of Timika

Photos: Anna Ralph

(a) Timika markets  (b) View from aeroplane flying south from Timika
6 AVDAPT Study design and methodology

In this methods chapter, I address Aim 1 (Chapter 1) of designing and commencing the AVDAPT study. The study protocol is registered online at http://clinicaltrials.gov/show/NCT00677339 (see Appendix 15.3). Information and consent sheets (English versions) and data collection forms are reproduced in Appendices 15.4 and 15.5.

6.1 BACKGROUND

The theoretical rationale for the AVDAPT study is provided in the introductory chapters of this thesis. Preliminary research at the Timika field site upon which this trial builds includes a study in 2003-4 of 115 people with pulmonary TB, examining risk factors for impaired lung function. Participants in this study had severe disease at diagnosis (as measured by sputum positivity, X-ray changes, lung function tests and functional measurements), low rates of HIV co-infection (4%) and MDR-TB (2%), high rates of malnutrition (41%) according to WHO criteria (BMI<18.5kg/m²), and a default rate of less than 10%. Thirty one percent of patients remained in the malnourished range at 2 months. Adjunctive therapy which could accelerate mycobacterial killing, modify immunological responses to TB and/or facilitate a more rapid return to normal weight in TB patients would be of great benefit in this population.

Preliminary results from trials of arginine supplementation in patients with malaria in Timika demonstrated substantial increases in exhaled NO: 6g supplementation led to a mean increase of 55%. The immunohistopathological changes associated with TB suggest that NOS2 expression in TB is at least as high as in malaria. Therefore, if the malaria result is replicated in the AVDAPT study, 6g of L-arginine would be expected to increase exhaled NO by at least 55%.

In unpublished data from Timika, median serum vitamin D concentration in 26 Non-Papuans was in the deficient range (<50nmol/L), at 45.5 nmol/L (range: 36-111).
Although Papuans (n=52) had significantly higher serum vitamin D (median 64.5, range 40-95, p=0.0003, Mann Whitney U test), 8% were still deficient, and vitamin D levels are likely to be lower still in those with active TB.

### 6.2 HYPOTHESES

The study hypotheses and aims set forth in Chapter 1 are reiterated as follows:

i. L-arginine supplementation in pulmonary TB will be safe, will increase plasma arginine concentrations, will enhance pulmonary production of nitric oxide (NO) (a key arginine-dependent immunomodulator and downstream immune mediator of mycobacterial killing) and will improve the rapidity and magnitude of the microbiological and clinical response. Baseline pulmonary NO production will be elevated in pulmonary TB but inversely associated with disease severity. Both baseline and post-treatment increments in exhaled NO will be associated with rapidity and magnitude of the treatment response.

ii. Supplementation with vitamin D, the metabolite of which (1,25-dihydroxyvitamin D₃) has anti-mycobacterial activity, will be safe, will increase plasma vitamin D concentrations, and will improve the rapidity and magnitude of the treatment response in human PTB.

### 6.3 AIMS

i. To determine whether supplementation with L-arginine and / or vitamin D is safe and results in more rapid improvement in clinical, mycobacterial, immunological, radiological, physiological and functional measures of treatment outcome. Patients with pulmonary TB will be randomised to receive, in addition to standard TB therapy, adjunctive L-arginine and vitamin D, singly or in combination, or matching placebos. Serial measurements of plasma concentrations of L-arginine and vitamin D will be examined in relation to immunological responses (pulmonary NO production, T cell function and phenotype) and measures of treatment outcome [mycobacterial (sputum smear clearance and culture conversion), physiological (spirometry), clinical (symptoms and weight),
radiological (chest Xray) and functional (six-minute walk test, modified St George Respiratory Questionnaire, SGRQ)).

ii. To determine whether exhaled NO (\(F_{ENO}\)) is inversely related to disease severity at presentation. Baseline and serial measures of NO production will be correlated with disease severity and the magnitude and rapidity of clinical response.

6.4 METHODS

SETTING

The study is being conducted at the Timika TB Clinic, Timika, Papua Province, Indonesia by the NIHRD-MSHR health research collaboration. Study personnel (Investigators, Consultants and members of the Data and Safety Monitoring Committee) are listed in Appendix 15.1.

STUDY POPULATION

Patients diagnosed with pulmonary TB in Timika who are referred for outpatient treatment to the Timika TB clinic are eligible for enrolment in the study. All patients with suspected TB are diagnosed according to the Indonesian National TB Protocol (NTP) guidelines. This comprises three sputum smears examined for acid fast bacilli (Ziehl Neelsen [ZN] stain on direct sputum specimens) in people with symptoms suggestive of pulmonary TB. Posteroanterior chest radiograph is also routine at TB diagnosis.

STUDY DESIGN OVERVIEW

The study has a factorial 2x2 study design (4 arms containing 2 interventions or their matching placebos). It is a randomised double-blind, placebo controlled trial of adjunctive oral L-arginine and / or vitamin D for 8 weeks, in addition to standard antituberculous treatment, for people commencing treatment for newly diagnosed with pulmonary TB (Figure 6.1). Additional to the randomised controlled trial (RCT), other related studies being undertaken in Timika are also shown in the Figure and described below.
Recruitment of healthy volunteers is described in Section 6.5. PK/PD measures are described in the section titled Evaluation of L-Arginine Pharmacokinetics and Pharmacodynamics.

Abbreviations: RCT: randomised controlled trial; 6MWT: 6 minute walk test, SGRQ: St George’s Respiratory Questionnaire, FE\textsubscript{NO}: fractional exhaled nitric oxide
INCLUSION CRITERIA

People aged 15 years or older are eligible if they have sputum smear positive pulmonary TB; have never received more that one month of anti-TB treatment in the past (that is, only new cases of TB are included); agree to continue treatment in Timika for the full six month course of treatment; are not pregnant, and consent to enrol in the study.

EXCLUSION CRITERIA

Eligible participants who consent but are subsequently found to have hypercalcaemia (ionized calcium >1.32 mmol/L prior to randomisation) are excluded from the study.

ENROLMENT PROCEDURE AND INFORMED CONSENT

All patients diagnosed with smear positive pulmonary TB who are commencing TB treatment are seen by the Timika TB clinic doctor (usually Dr Andri Wiguna). A log of all such patients commencing treatment, their eligibility for the study, and reasons if any for non-enrolment, is maintained by the TB clinic doctor and research assistant (RA) staff. All apparently eligible patients are referred to one of the three AVDAPT RAs, whose workspace is in the adjoining room (see diagrammatic summary of enrolment procedure, Figure 6.3). The RAs (2 nurses, Govert Waramori and Gertruida Bellatrix and 2 doctors, Gysje Pontororing and Enny Kenangalem) are employed by or seconded to the NIHRD-MSHR Health Research Collaboration, and have received specific training and supervision in all aspects of the study protocol. After being referred a potential study participant, they conduct a thorough eligibility assessment, and seek informed consent from eligible patients.

To obtain informed consent, patients receive a detailed verbal and written explanation regarding the reason for the study and what it involves, and regarding the voluntary nature of involvement in the study. The written information sheet is provided in Bahasa Indonesia (English version: Appendix 15.4). Verbal explanations are provided using a Papuan interpreter where required. A pictorial aid (Figure 6.2) is used to assist the consent process. Written consent is obtained using the signature or thumbprint of the participant, and of the parent or guardian for participants aged between 15 and 18.
Figure 6.2: Consent booklet

Penelitian TBC “ADAPT”
Informasi

Information about TB ‘AVDAPT’ study

Diskusi dan kuisioner

Discussions and completing questionnaires

Ambil darah

Take blood at week 0, 1, 2, 4, 8, 24

Pemeriksaan darah

Blood tests include calcium measurement, and HIV testing if specific additional consent is given

Tes fungsi paru ‘nitric oxide’

Exhaled nitric oxide is measured at week 0, 1, 2, 4, 8, 24

Tes fungsi paru ‘volume’

Pulmonary function tests are performed at week 0, 4, 8 and 24
Chapter 6: Methods

Six minute walk test is performed at week 0, 4, 8 and 24. Chest x-ray is performed at week 0 (prior to enrolment in study) and again at week 8 and 24.

Standard TB medications are taken as fixed-dose combination (number of tablets determined by body weight). Study medications (L-arginine &/or vitamin D &/or matching placebos) are taken for the first 8 weeks.

Follow-up setiap minggu selama 8 minggu, kemudian minggu ke 12, 16, 20, 24.

Study participants must attend at least weekly for the first 8 weeks, then monthly during the continuation phase of treatment.

Only patients who understand the study and sign the consent form are enrolled. It is explicitly stated that involvement is voluntary, participants are able to say no, and if they do initially consent, they can leave the study at any time without prejudicing their treatment in any way. On withdrawing from the study, the researchers provide ongoing...
positive encouragement and support for the participants to continue her/his TB treatment as usual, according to the NTP.

Consenting participants have blood drawn for point-of-care calcium measurement (ionised calcium using iSTAT® device). Hypercalcaemic patients (confirmed on 2 tests) are excluded from the study as per the exclusion criteria, and referred to the TB clinic doctor for ongoing management. Normocalcaemic patients are retained in the study, and are assigned to an unidentified (blinded) study arm, by receiving a sequential, unique study identification number stratified by ethnicity, printed on an opaque envelope containing the study medications (see also section below, ‘Randomisation and Blinding’). Baseline evaluations outlined below are performed, and the first supervised dose of TB medications and study drugs is administered. All study participants are requested to attend the clinic daily or weekly during the intensive phase, and monthly during the continuation phase to receive their anti-TB medications, study medications, routine examinations and the study examinations as outlined below. If patients do not attend daily, a family member is requested to act as the medication supervisor on non-clinic days.

**TB treatment regimen**

The standard TB treatment regimen used in Timika is rifampicin (10mg/kg, max 600mg), isoniazid (10mg/kg, max 300mg), ethambutol (15mg/kg) and pyrazinamide (25mg/kg, max 2g) 7 times weekly during the intensive phase (2 months, or 3 months if smear status still positive at 2 months), then rifampicin (15mg/kg, max 600mg) and isoniazid (15mg/kg, max 600mg) 3 times weekly (continuation phase, 4 months). These antibiotics are compounded in fixed dose combinations (e.g. one intensive phase tablet contains rifampicin 150mg, isoniazid 75mg, ethambutol 275mg and pyrazinamide 400mg), allowing close approximations of the recommended doses per kg to be achieved by following standard guidelines on number of tablets to be taken per day according to weight.

**Study medication regimens**

Study medications, administered separately from the pre-packaged TB medications, are allocated to the 4 study arms in the following way:
• Group 1: supplementary L-arginine 6g daily for eight weeks plus vitamin D₃ (cholecalciferol) 50 000 IU (1250mcg) once at baseline and once on day 28 (0 and 4 weeks);
• Group 2: supplementary L-arginine 6g daily for eight weeks plus placebo vitamin D₃ once at baseline and once on day 28 (0 and 4 weeks);
• Group 3: placebo L-arginine daily for eight weeks plus vitamin D₃ (cholecalciferol) 50 000 IU (1250mcg) once at baseline and once on day 28 (0 and 4 weeks);
• Group 4: placebo L-arginine daily for eight weeks plus placebo vitamin D₃ once at baseline and once on day 28 (0 and 4 weeks).

Figure 6.3: Enrolment procedure

1. Diagnosis
Patient diagnosed with smear positive pulmonary TB (clinical suspicion of TB and at least 2 AFB positive sputum specimens). TB doctor refers to AVDAPT research assistant (RA).

2. Eligibility screening:
RA assesses whether referred patients fulfill inclusion criteria.

3. Consent process:
Written, pictorial and verbal information provided about the study, with Papuan interpreter if necessary. Patient indicates consent by signing or providing thumbprint.

4. Blinded assignment to study arm / Allocation of study code:
A unique, sequential study code, stratified by ethnicity, is allocated. The code comprises the letters TAD (TB, Arginine, Vitamin D) and a 4 digit number, starting with 1 for Papuans or 2 for Non-Papuans.

5. Allocation of medication pack:
The study medications for the full 8 week course are allocated. They are contained in zip-lock bags inside a large, sealed, opaque envelope, identified with the study code.
L-arginine hydrochloride formulation and dose

L-arginine hydrochloride tablets (Argimax®) used in the AVDAPT study are manufactured by Hankintatukku Oy, Finland according to Good Manufacturing Practice (GMP) standards, in 1g tablets packaged in blister packs (Certificate of Analysis, Appendix 15.7). Tablets are large, oblong, uncoated pills. Matching placebo tablets containing microcrystalline cellulose, maize starch and magnesium stearate, have been purposely manufactured for the AVDAPT study by the same manufacturer with identical appearance, colour, size, taste and packaging as active Argimax® tablets.

The choice of 6g daily is based on clinical experience with this dose (see Chapter 2), safety profile at this and higher doses, and the preference for a once daily regimen for TB patients to avoid dosing errors (as other TB medications are dosed once daily), and to optimise adherence.

Vitamin D3 (Cholecalciferol) formulation and dose

Cholecalciferol (Calciferol Strong®) is manufactured by API Consumer Brands, New Zealand, in 50 000 IU (= 1250 mcg) doses, packages in sterile bottles. They are manufactured according to GMP (Certificate of Analysis, Appendix 15.8), and glycerine used in the tablets is manufactured according to Halal requirements, as required for use in Indonesia. Tablets are small, round, coated white tablets, easily distinguishable from the L-arginine / L-arginine placebo and TB pills. A batch of placebo tablets identical in every way to active cholecalciferol tablets has also been specifically manufactured for this study by API Consumer Brands.

The dose of 50 000 IU once monthly for 2 months has been selected to provide adequate replacement for those who are vitamin D deficient, and avoid toxicity in those replete at baseline (see Chapter 2).

Preparation of study medication packs

Study medications are pre-labelled and packaged by an unblinded MSHR researcher (Ms Kim Piera, who is not involved in any clinical aspects of the study), according to the random allocation sequence.
• **Argimax® / placebo blister packs**
  Each blister pack is labelled with a sticker containing all information required according to Good Clinical Practice (GCP) regulations (study participant’s code, space for investigator to write name and date, drug information, study information. See sample sticker, Figure 6.4). Enough tablets for one week (42 tablets, 6 per day) are packaged into each of 8 zip-lock bags, labelled week 1-8.

• **Calciferol Strong® / placebo tablets**
  One tablet is placed in a small zip-lock bag labelled with a sticker, similar to Figure 6.4 but stating that the medication is vitamin D or vitamin D placebo. One labelled, individually packaged tablet is placed in the week 1 study medication bag, and one in the week 5 study medication bag.

The clearly marked Study Drug Packs are stored in an air-conditioned room at the Timika TB Clinic until being assigned to a study participant, at which stage they are transferred to a refrigerator.

**Figure 6.4: Sample stickers applied to study medications**

(a) Indonesian

LITBANGKES-MSHR PENELITIAN AVDAPT

KODE STUDI: TAD-1001 TANGGAL: ___|___| 20____

Nama_____________________________________

DOSE minum obat 6x tablet bersamaan dengan makanan setiap haro. Tablet ini bersini arginine 1g atau placebo. Pelajaran no. 0740. Emergensi kontak: Dr Daniel 0811 491 699 MASA BERLAKU: Maret 2011

(b) English

LITBANGKES-MSHR AVDAPT STUDY

STUDY CODE: TAD-1001 DATE: ___|___| 20____

Name_____________________________________

DOSE take 6x tablets daily with food. These tablets contain arginine 1g or placebo. Study no. 0740. Emergency contact: Dr Daniel 0811 491 699 MEDICATION EXPIRY DATE: March 2011

**PARTICIPANT EVALUATIONS**

**Height, weight and BMI**

Standing height is measured without shoes to the nearest centimetre using a stadiometer.
Weight in kilograms is measured without shoes to the nearest 0.1kg using an adult balance. BMI is calculated in the standard way, i.e: \( \text{BMI} = \frac{\text{weight (kg)}}{\text{height (m)}^2} \)

**Questionnaires**

An enrolment questionnaire is completed once per patient, and a regular questionnaire (Appendix 15.5) is completed weekly during weeks 0 to 8, then monthly until treatment completion. All data collection forms and other documents are labelled with the participant’s study code. The regular questionnaire assesses medication adherence,
results of point-of-care blood tests (haemoglobin and calcium), clinical measures (lung function, exhaled nitric oxide, 6 minute walk test, lung function tests), symptoms including cough (mild, moderate, severe or very severe, as subjectively judged by the patient), and any adverse events. Any medications additional to TB and study medications are documented, and whether HIV counselling and testing has been performed is recorded.

**Chest radiograph**

A posteroanterior chest x-ray is performed as per standard clinical protocols at the TB clinic prior to patient enrolment in the study, and at week 8 and 24. Chest x-rays are reported by myself, using the method described in Chapter 9. Study arm allocation concealment is maintained at all times.

**Pulmonary Function**

Pulmonary function is measured using a handheld spirometer (ML3535C MicroLoopTM, Micro Medical, UK), which is calibrated daily using a standard 3L syringe (Figure 6.5). Individual-use filtered one-way mouthpieces suitable for use in smear-positive pulmonary TB patients are used (Sure-Gard®). Each patient performs at least three maximum effort expirations until volumes vary less than 200mls, with the highest values for FVC and FEV$_1$ used as measures of lung function. RAs receive training and supervision in use of the device initially, and on a regular periodic basis thereafter. Measurements are performed outside (in the area adjacent to the TB clinic).

**Exhaled nitric oxide**

Fractional exhaled nitric oxide (FeNO), described in Chapter 3, is used to evaluate pulmonary NO production, using a portable online device, the NiOX MINO® (Aerocrine, Sweden). This device is well validated, easy to use, does not require calibration, and employs single-use disposable, filtered mouthpieces. The process does not pose any infection control risks. FeNO is measured prior to spirometry and the 6 minute walk test, and subjects are requested to avoid eating or vigorously exercising for at least one hour before testing. Measurements comply with ATS 2005 Guidelines. To assess for sensor drift or inter-sensor variation, quality control (QC) measures
comparing results from biological controls (RA staff) are compared on a weekly basis between the NiOX MINO® and a ‘gold standard’ non-portable NiOX FLEX®.

**Figure 6.5: Spirometer calibration using 3 litre syringe**

*Research assistant Bapak Govert Waramori at the Timika TB clinic*

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**Modified St George Respiratory Questionnaire (SGRQ)**

The SGRQ is a health-related quality of life instrument formulated for use in chronic obstructive pulmonary disease. The English version is supplied in Appendix 15.6. An overall score (range 0-100) and individual domain scores for symptoms, activity and impacts on daily life are obtained. Low scores indicate better lung health.

An Indonesian translation of the SGRQ was provided by the creators of the questionnaire (Professor P.W. Jones, St George’s Hospital Medical School, London, United Kingdom), and slightly modified to reflect local conditions in Timika. Results of this modified SGRQ have previously been reported in TB patients in Timika. Standard recommendations are that patients complete the SGRQ without assistance; however given literacy rates at the field research site, the trained RAs administer the questionnaire in an impartial manner, and document participant responses.
6 minute walk test (6MWT)

The 6 minute walk test is a practical, simple test measuring the distance a patient can quickly walk on a flat, hard surface (ideally 100 ft i.e. 30.5m in length) in a period of 6 minutes. It is widely used as a measure of functional exercise capacity. Multiplying the 6 minute walk distance by body weight to generate weight · walk distance has been shown in some studies to correlate more closely with energy expenditure and lung function, and hence 6 minute weight · walk distance is also calculated.

6MWT is assessed in study participants on a 20m open air path beside the TB clinic. Conduct of the 6 minute walk test adheres to ATS guidelines, with the exception that a minimum 30m track length is preferred, although use of 20m tracks in other studies has not resulted in any significant difference in distance walked. The 20m track length is the maximum available at the study site, is consistent throughout the study, and healthy volunteers are tested on the same track to establish a comparative normal range (see below).

Sputum collection and processing

Prior to enrolment in the study, smear positive TB is diagnosed at the TB clinic (or at another community clinic or hospital outpatient centre, with subsequent referral to the TB clinic), on the basis of ≥2 sputum specimens positive acid fast bacilli (AFB), in accordance with NTP standard protocols.

Additionally on the day of entry into the study, a further sputum specimen is obtained, results of which are recorded as the study participant’s baseline AFB grade and culture result. Follow up samples are collected weekly up to week 8 for smear microscopy at the on-site laboratory. Mycobacterial culture facilities are unavailable throughout Papua Province. At weeks 0, 4 and 8, samples are therefore additionally dispatched for culture and drug susceptibility testing (DST) to the University of Indonesia Faculty of Microbiology (FKUI), Jakarta (see Table 6.1).

At the on-site laboratory at the Timika health clinic (Puskesmas) complex, a direct slide is prepared under an approved biological safety hood, and examined under a light microscope using standard ZN stain. WHO guidelines for grading smear results are
adhered to. Microscope slides labelled with the study code and date are stored at the NIHRD-MSHR Research Building in Timika for later quality control testing.

Samples collected at weeks 0, 4 and 8 are divided for use (in some instances 2 simultaneously-collected samples have been used); after slide preparation at the local laboratory, the sample is stored (batched for up to 2 weeks) in a 4°C refrigerator, then packaged by the RA (Govert Waramori) who is trained in International Air Transport Association regulations, and dispatched unrefrigerated by courier to FKUI. Unrefrigerated transportation of fresh sputum samples has previously been shown by MSHR researchers to be associated with good MTB recovery rates. At FKUI, slides prepared directly and from concentrated sputum are examined using the ZN method as above. Concentration is achieved by adding 4% sodium hydroxide, repeatedly homogenising the mixture using a vortex, adding phosphate-buffered saline, then obtaining the concentrated sample by centrifugation.

Culture is performed at FKUI for each specimen using both liquid and solid media. Sputum specimens are decontaminated using 2% sodium hydroxide and 0.5% N-acetyl-cysteine for 25 minutes, then neutralised to pH 7, concentrated by centrifugation (3,000xg for 15 min) and inoculated into a single Mycobacterium Growth Indicator Tube 960 (MGIT; Becton Dickinson Microbiology Systems, Sparks, Md) and two tubes of Lowenstein-Jensen media. DST is performed on week 0 specimens, using the BACTEC MGIT 960 TB system, using the recommended critical concentrations for the following antibiotics: isoniazid (0.1), rifampicin (1.0 mg/ml), ethambutol (5.0 mg/ml), streptomycin (1.0 mg/ml). In instances of multi-drug resistance, second line testing to ofloxacin, amikacin and kanamycin is performed.

Confirmation of MDR-TB or rifampicin monoresistance is achieved using the Hain GenoType® MTBDRplus assay. This assay permits the molecular genetic identification of MTB complex, and detects the most common mutations occurring within the rpoB gene that confer rifampicin resistance, and the mutations conferring low-level isoniazid resistance (inhA gene) and high-level isoniazid resistance (katG gene). DNA is extracted from cultured MTB isolates, followed by multiplex amplification and reverse hybridization steps as per the manufacturers instructions. Results are provided on paper strips with visible lines indicating the presence of wild type or mutation genes.
Figure 6.6: Mycobacterial growth indicator tubes (MGIT)
MGIT inoculated with MTB containing (L-R): ethambutol, rifampicin, isoniazid, streptomycin, no antibiotic (control tube), for determination of drug susceptibility
(Photo: Anna Ralph, taken at FKUI, Jakarta).

**FOLLOW UP**

Study participants are re-evaluated during the 6 month follow up period according to the schedule in Table 6.1 (tests are performed when indicated by a tick). Default tracing, including telephoning or text messaging for participants with telephones, visiting patient’s homes and contacting neighbours or relatives, is conducted rigorously to attempt to trace participants who miss scheduled clinic appointments.

Table 6.1: Follow-up schedule for AVDAPT study participants

<table>
<thead>
<tr>
<th>Test</th>
<th>Week of TB treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Weight</td>
<td>✔</td>
</tr>
<tr>
<td>Symptoms</td>
<td>✔</td>
</tr>
<tr>
<td>Sputum microscopy*</td>
<td>✔</td>
</tr>
<tr>
<td>Sputum culture</td>
<td>✔</td>
</tr>
<tr>
<td>Chest x-ray</td>
<td>✔</td>
</tr>
<tr>
<td>Spirometry</td>
<td>✔</td>
</tr>
<tr>
<td>Exhaled nitric oxide</td>
<td>✔</td>
</tr>
<tr>
<td>6 minute walk test</td>
<td>✔</td>
</tr>
<tr>
<td>SGRQ</td>
<td>✔</td>
</tr>
</tbody>
</table>

*Sputum microscopy is repeated weekly if still positive at week 8, until negative. †If the patient has not completed treatment by week 24, a further follow up as for week 24 is repeated at treatment completion.
Blood is collected from participants at weeks 0, 2, 4, 8 and 24. Assays, tube types and volumes are indicated in Table 6.2.

<table>
<thead>
<tr>
<th>Blood test</th>
<th>Tube</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV*</td>
<td>3ml EDTA†</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parathyroid hormone</td>
<td>3ml EDTA</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>25(OH)D₃, 1,25(OH)₃D₃ and amino acids</td>
<td>6mL LiHep‡</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Calcium and haemoglobin</td>
<td>6mL LiHep</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripheral blood mononuclear cells and Interferon gamma release assay</td>
<td>10mL LiHep</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
<td>✔</td>
</tr>
<tr>
<td>Back-up serum for storage</td>
<td>2mL LiHep</td>
<td>✔</td>
<td></td>
<td></td>
<td></td>
<td>✔</td>
</tr>
<tr>
<td>Total blood volume</td>
<td>19ml 18ml 19ml 6ml 18ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*HIV is ideally performed at week 0 but may be deferred depending on pre-test counselling availability. †EDTA = ethylene diamine tetra-acetic acid blood tube; ‡LiHep = Lithium heparin blood tube

Methods for on-site blood processing (i.e. in Timika):

- HIV: Standard Diagnostic (SD)® point-of-care HIV test on plasma or whole blood. If positive, confirmation is performed using Abbott Determine® and Oncoprobe® point-of-care tests. In instances where SD kits have been unavailable, FOKUS® test kits have been used. All point-of-care tests have high sensitivity and specificity (e.g. Standard Diagnostic (SD)®: sensitivity 100%, specificity 99.8%). Written, informed consent is obtained as part of routine TB using the Indonesian national standard ‘Voluntary Counselling and Testing for HIV’ form, by either the TB clinic doctor, AVDAPT RA (doctor), or on-site sexual health clinic doctor or trained counsellor.

- Ionised calcium and haemoglobin: point-of-care iSTAT cartridges and iSTAT analyser. New batches of iSTAT cartridges are tested using standard QC vials.

- Blood film, cell count and differential: A blood film is prepared for manual white cell count and differential

- Interferon gamma release assay: Interferon-γ production in response to stimulus with mitogens, bacterial LPS and PPD will be determined using ex vivo Quantiferon® assays. These will performed when blood is available from all required time points (weeks 0, 2, 4, 24), for testing in parallel.
Methods for storage of blood prior to processing in Australia:

- Peripheral blood mononuclear cell (PBMC) separation according to MSHR protocols. Samples are stored at -70°C (plasma) or in liquid nitrogen (PBMCs in foetal calf serum/DMSO) in the Timika research laboratory, for transportation by dry shipper at -70°C to MSHR.

Methods for blood processing in Australia:

For reasons described in Box 6.1, samples are currently in storage and are yet to be transported to Australia for analysis. Planned analysis methods are as follows:

- 25(OH)D₃: isotope-dilution liquid chromatography-tandem mass spectrometry (LC-MS) method, RMIT Drug Discovery Technologies Pty Ltd, Victoria
- 1,25(OH)₂D₃: IDS Radioimmunoassay, IMVS
- Parathyroid hormone (PTH): IMMULITE 1000 Intact PTH®, IMVS
- Quantitative L-arginine, citrulline and ornithine: High performance liquid chromatography, MSHR
- White cell studies (PBMC phenotype, CD3 zeta expression in activated and naïve CD4 and CD8 T cells): will be determined using fluorescence activated cell sorting (FACS). Phenotypic analyses will be run in conjunction with functional assays to measure cell proliferation and cytokine secretion. Cell proliferation will be detected by using carboxyfluorescein diacetate succinimidyl ester (CFSE).
**EVALUATION OF L-ARGININE PHARMACOKINETICS AND PHARMACODYNAMICS**

In a sub-set of 60 sequential patients who give additional verbal consent to wait at the clinic for up to 4 hours and have repeated venesection, the 18mL blood collected at week 2 is obtained in three divided aliquots before and up to 4 hours after dosing with study medications, accompanied by repeated \( \text{FE}_\text{NO} \) measures over the same time period, in order to:

- Determine the pharmacokinetic profile of orally-administered L-arginine 6g by evaluating time to maximum plasma concentration (Tmax) and plasma half life, and
- Determine the pharmacodynamic profile of orally-administered L-arginine 6g by evaluating time to peak and normalization of \( \text{FE}_\text{NO} \).

The times of blood draws, \( \text{FE}_\text{NO} \) measurements and tablet dosing are accurately recorded on a data collection sheet (Appendix 15.5).
Box 6.1: Explanation regarding planned off-shore laboratory analyses

Prior to commencement of the AVDAPT study, Materials Transfer Agreements were signed by the Indonesian and Australian directors of the NIHRD-MSHR collaboration, and directors of the respective laboratories where assays were to be performed. These agreements included that the originating country (Indonesia) would be acknowledged in any publications arising from sample usage, and that there would be no further distribution of specimens outside the laboratories specified in the agreement.

However due to a subsequent directive from Indonesia’s 2004-09 Minister of Health, Dr Siti Fadilah Supari, biological samples collected from AVDAPT study participants were prevented from being exported for analysis outside Indonesia, coinciding with the date of study commencement (June 12th 2008). Arising from grievances over usage of Indonesian H5N1 virus samples which had been shared with WHO in 2005, Indonesia claimed that biological samples are its “sovereign property and do not constitute resources that other countries or the international community can access and use”.

Ramifications have included threats to close U.S. Naval Medical Research Unit 2 (NAMRU-2), a public health laboratory in Jakarta, and describing NAMRU-2 scientists as “profiteering off its ‘sovereign’ viruses to manufacture the H5N1 bird flu in an alleged biological warfare scheme.” Dr Supari’s concern that the US might be involved in a conspiracy to use the bird flu virus to develop biological weapons is also expressed in her book (endorsed by Indonesian President Yudhoyono) entitled It's Time for the World to Change: Divine Hands behind Bird Flu (Saatnya Dunia Berubah: Tangan Tuhan dibalik Flu Burung). Dr Supari and colleagues at NIHRD articulated the perceived unethical outcomes of sharing of biological samples in an article published in the Annals of the Academy of Medicine Singapore in 2008 as follows:

“Disease affected countries, which are usually developing countries, provide information and share biological specimens/virus with the WHO system; then pharmaceutical industries of developed countries obtain free access to this information and specimens, produce and patent the products (diagnostics, vaccines, therapeutics or other technologies), and sell them back to the developing countries at unaffordable prices.”

Indonesia’s stance on withholding H5N1 virus samples from WHO and potential vaccine developers eventually led to a World Health Assembly resolution being adopted on May 23, 2007, stipulating that “transparent, fair and equitable sharing of the benefits arising from the generation of information, diagnostics, medicines, vaccines and other technologies” would be promoted. Indonesia thereafter resumed sharing influenza virus samples, but has remained protective of biological samples in general.

Ramifications for the AVDAPT study are that to date, those assays that were approved by the Indonesian Ministry of Health Ethics Committee (and the NIHRD Ministry of Health Materials Transfer processes) for processing in Australia are yet to be performed. The study protocol has been adapted where possible to accommodate the regulation. Rather than undertaking sputum culture and DST at the WHO supranational reference laboratory (IMVS) in Australia as planned, sputum is now processed at FKUI, Indonesia (this laboratory was fortuitously accredited for mycobacteriological culture by WHO in May 2008). The capability for performing the blood assays designated for processing in Australia, to Good Laboratory Practice recommended standards, is not currently possible in Indonesia. Therefore samples are stored at -70 degrees as described, for transfer for processing when this regulation has been amended.
TRIAL OUTCOME MEASURES

A number of outcome measures will be evaluated. The primary outcome measures are:

- Proportion of pulmonary TB patients who are culture negative at 1 month.
- Difference in improvement in composite clinical endpoint comprising weight, cough clearance and FEV$_1$ at 2 months.

The secondary outcome measures are:

- Safety.
- Difference in improvement in percent predicted FEV$_1$ at 2 and 6 months.
- Change in plasma L-arginine and 25(OH)D$_3$ concentrations.
- Weight gain.
- Cough clearance.
- Sputum smear conversion time.
- Functional improvement including Six minute walk test and quality of life assessment using modified St George’s Respiratory Questionnaire.
- Immunological improvement (exhaled NO, T cell CD3ζ expression and T cell function).
- Radiological improvement (percentage lung involvement on CXR at 2 months).
- Percentage obstructive and/or restrictive lung disease at 6 months.
- Death, clinical failure and default independently, and ‘death or clinical failure or default’.
- Primary end points stratified by HIV status.
- Primary end points stratified by baseline vitamin D and L-arginine status.
- Primary end points stratified by ethnicity (Papuan and non-Papuan patients).

Use of mycobacterial culture as a primary outcome measure overcomes the potential problem of continued smear positivity of non-viable organisms. Although various outcomes have been selected by TB researchers as surrogates for 6-month treatment outcome and 2-year relapse, including 2-month sputum smear (a predictor of treatment failure$^{142-144}$) or 2-month sputum culture conversion,$^{145, 146}$ culture at one month has been selected in this study in order to capture any early microbiological effect of adjunctive therapy.
The composite clinical outcome score, assigning weights to percentage improvement in weight gain and FEV1 improvement, and to respiratory symptoms (cough, sputum production and haemoptysis), is described in detail in Chapter 10.

**STATISTICAL ANALYSES**

**Sample size**

Because of the potential for interaction when 2 interventions are trialled in a single study, it is recommended that sample size in a 2x2 factorial study be calculated by estimating the size of any potential interaction, then using the Fleiss equation.\(^\text{147}\) Making a conservative assumption of sub-additive interaction between L-arginine and vitamin D (interaction coefficient of 0.5), then at a level of significance of 5%, a sample size of 444 (111 participants in each arm) was found using this method to be appropriate, providing 82% power to demonstrate that each treatment results in a 20% reduction in the proportion culture positive at one month (from 60% to 40%), assuming loss to follow up of 10%. As there have been no trials of co-administration of vitamin D and L-arginine, the characteristics of potential immunological interactions can be extrapolated only from *in vitro* macrophage studies, in which findings conflict: vitamin D was found to inhibit NOS2 expression in one study,\(^\text{148}\) yet upregulate it in another.\(^\text{149}\) The latter study also found the suppressive effect of vitamin D on MTB replication to be partially impaired if NO formation was inhibited. Including a sub-additive interaction term in the sample size calculation provides a margin a caution; if there is minimal or no interaction between the two interventions, power will be greater. A factorial design rather than a three-arm study allows investigation of whether it is safe and effective to co-administer the two interventions. Acknowledging the possibility of statistical interaction between the terms, the *a priori* plan is to analyse primary and secondary outcomes according to subgroups receiving single interventions, as well as according to the overall factorial model.\(^\text{147}\)

Mean time to culture negativity in mostly drug sensitive TB has been reported to be 32 days (New York)\(^\text{150}\) to 57 days (Turkey).\(^\text{151}\) Based on prior observations of high bacillary burden and extent of cavitary disease in Timika TB patients, a minimum of 60% of Timika patients are estimated to be culture positive at 1 month. Power will be increased if this proportion is higher. This sample size will also be powered to detect a
9% absolute difference in the mean improvement in percent predicted FEV$_1$, a component of the composite endpoint, at 6 months. A previous study in Timika found mean baseline percent predicted FEV$_1$ to be 63.6% predicted (SD 22) rising to 77.9% predicted (SD 23) at 6 months.\footnote{114}

**Outcome Analysis**

Data is double-entered and validated using EpiData 3.02 software (EpiData Association, Odense, Denmark) and analysis will be performed using Stata software (version 10.1; Statacorp) and GraphPad (version 5.0; Prism). An *a priori* analytical plan will be followed for evaluation of trial outcomes. The two primary outcomes (sputum culture positivity at one month and composite clinical outcome score at two months) will be determined by an independent observer, blinded to the intervention arm, and compared on an intention to treat basis. Descriptive statistics will be presented for all outcomes for each of the 4 factorial cells in the trial (L-arginine, vitamin D, L-arginine plus vitamin D, and placebo). We will assess the main effects of the 2 interventions (L-arginine versus no L-arginine and vitamin D versus no vitamin D) by estimating the average effect on the primary endpoints. In the primary analysis, results will be presented as adjusted odds ratio (and 95% confidence intervals [CI]) and mean difference in change in clinical score (and 95% CI). Regression models (logistic regression or multiple regression/analysis of variance) will be used to adjust for co-interventions, ethnicity and HIV status. Any interaction between the interventions will also be described (with its 95% CI). Secondary endpoints listed above will be assessed on a per protocol population. Time to smear negativity will be assessed by survival analysis using the Kaplan Meier method. Changes in plasma L-arginine, 25(OH)D$_3$ and exhaled NO will be compared between treatment arms, and primary and secondary outcomes will be related to baseline pulmonary NO production/L-arginine/25(OH)D$_3$ and clinical, radiological, microbiological and functional measures of disease severity at enrolment.

**Assessment of Safety**

**Adverse events**

As per GCP guidelines, adverse events (AE) are defined as any new signs or symptoms reported or observed during the course of the study which were not evident at baseline. Serious adverse events (SAE) are defined as death, life-threatening illness or
hospitalisation. Adverse events may be unrelated to the study, may be a consequence of TB per se or TB treatment, or may be a consequence of the study interventions. Adverse event reporting guidelines are reproduced in Appendix 15.9.

SAE are notified immediately to study investigators (usually myself) and a case summary is prepared the form supplied in Appendix 15.10. I then submit this report to the clinician members of the Data and Safety Monitoring Committee, who determine the likelihood of relatedness of the SAE to the study medications (unrelated, unlikely, possible, probable or definite, as per GCP definitions shown in Appendix 15.9). Non-serious Adverse Events (AE) are managed by the study investigators, RAs and the local TB clinic doctor as required, and are reported to the Data and Safety Monitoring Committee at the time of interim analyses.

AE from oral L-arginine are uncommon. Any potential adverse effects of L-arginine are carefully monitored. Use of up to 21g daily was not associated with adverse events in two studies. Oral L-arginine 7g three times daily for 4 weeks was associated with diarrhoea in 3 of 27 patients, but this resolved on dose reduction to 7g twice daily. Minor gastrointestinal discomfort has been reported in up to 3% of patients. Adverse events potentially attributable to L-arginine in this study include diarrhoea, bloating, nausea, vomiting, mild to moderate abdominal pain.

Although much less common than in other granulomatous diseases, vitamin D supplementation in TB has the potential for hypercalcaemia (iCa^{2+} > 1.32 mmol/L) from extra-renal 1,25(OH)_{2}D_{3} production. Based on clinical experience with vitamin D supplementation (Professor John Eisman, listed study consultant as per Appendix 15.1), and review of the literature, the risk of symptomatic hypercalcaemia as a complication of vitamin D supplementation at the dose used in this study in patients with pulmonary TB is anticipated to be very low. Ionised calcium is measured, using point-of-care iSTAT testing providing immediate results, at 0, 1, 2, 4 and 8 weeks. In the event of hypercalcaemia developing, the literature indicates that the most likely time of onset would be 2-4 weeks post commencement of anti-TB therapy. Potential symptoms of hypercalcaemia, including nausea, vomiting, abdominal pain, constipation, anorexia, fatigue, irritability, confusion, somnolence, coma and dehydration, are checked for on a weekly basis. Study participants with hypercalcaemia are managed as
per the flow chart (Appendix 15.11), including having vitamin D / vitamin D placebo withheld.

Data and Safety Monitoring Committee

The Data and Safety Monitoring Committee has Indonesian and Australian membership comprising experienced researchers and health practitioners independent of the research team: Dr Paulus Sugiarto, Clinical Director, RSMM Hospital, Timika (chair of the committee), Dr Louise Maple-Brown, Specialist Physician and Endocrinologist, MSHR, Dr Joseph McDonnell, Statistician, MSHR and Ibu Hapsari, Statistician, NIHRD. The clinicians (Dr Sugiarto and Dr Maple-Brown) provide reports on any SAEs and advise whether any action to address the SAE is required by the study investigators, and whether they deem the study to be safe to continue. Interim safety analyses are conducted by the statistician members of the committee (Dr McDonnell and Ibu Hapsari) at time points as indicated below. The Data and Safety Monitoring Committee has the power to immediately discontinue the study if severe adverse events attributable to the study medications occur.

Stopping rules

Interim safety analyses will be performed after 25% and 50% of participants have been followed to 2 months (first analysis complete, see Chapter 10), or as requested by the Data and Safety Monitoring Committee in the event of SAE related to study drugs. The committee will be able to recommend that enrolment into the study ceases if they believe there is evidence that individuals are being harmed through their participation in the study. Early termination of the study (or of one of the study arms) may also be recommended if there is strong evidence of benefit or harm associated with either of the interventions at the interim analysis (p<0.01) or if there is strong evidence that further enrolment will not provide any useful information.

Randomisation and Blinding

A random allocation sequence assigning participants to an intervention group was computer-generated (Stata 9.2) by an independent statistician and will remain concealed from all investigators throughout the study. Block randomisation was used to maintain
similar numbers of participants in the intervention and control groups and to minimise the potential influence of time of enrolment. Allocation is stratified by place of TB enrolment (hospital or clinic, but enrolments have only taken place at one study site [clinic] to date) and ethnicity (Papuan/Non-Papuan), the latter because of the different Vitamin D status of these two groups (see ‘Background’ at the start of this chapter). Allocation concealment conforms to the revised CONSORT guidelines which take account of the logistically challenging environment of the study site.\textsuperscript{161} This method has been used successfully in over 1,200 malaria patients (three RCTs) in Timika.\textsuperscript{162-164}

The randomisation number corresponds to a study medication pack in a sealed opaque envelope, comprising the 2-month supply of study medications (as described above). Medications inside the envelope are indistinguishable as active or placebo medications in all respects. The unique code is used to identify all documents and samples for that study participant. The code comprises the letters TAD (TB: Arginine and Vitamin D) to distinguish the study from other studies being conducted at the field research site, and a unique 4-digit code (commencing TAD-1001 for Papuans and TAD-2001 for Non-Papuans). Local RAs ensure that patients have been randomised in the correct manner set out in this protocol. Any enrolment violations are clearly documented and notified to myself or other study investigators.

Only the study statistician and the Data and Safety Monitoring Committee see any unblinded data, and these individuals do not have contact with study participants. The randomisation code will be kept at MSHR by an independent person unrelated to the study. The randomisation sequence will not be broken until primary outcomes have been measured and agreed by the study investigators.

\textbf{ETHICAL CONSIDERATIONS}

\textit{Ethics committee approvals}

This study has been approved by the Human Research Ethics Committees of Menzies School of Health Research, the National Institute for Health Research and Development, and the Australian National University.
**Ethical considerations of randomised design**

The withholding of a potentially beneficial medication to half the enrolled study participants might be perceived as an ethical consideration. To alleviate these concerns, AVDAPT investigators explain clearly to staff, community leaders and patients: (a) the necessity for the blinded, randomised, placebo-controlled approach, and (b) the fact that there is as yet no evidence of benefit of L-arginine and/or vitamin D supplementation in TB, and hence those receiving placebo will not necessarily be disadvantaged. The verbal and written information provided by local research assistants and interpreters explain that half the patients enrolled will not get the active study drug. Patients are reminded that they can withdraw from the study at any time without compromising their care or relationship with clinic staff. MSHR researchers have successfully employed blinded RCT design in the local population in large malaria trials to date, with patients willing to participate in such a study design.

All patients, including those who decline to participate in the study, continue to receive all aspects of standard TB care. They may in fact experience improved care due to greater attention being given to the importance of TB medication adherence, quality control aspects of the study which will also have benefits for patients not enrolled in the study, and the presence of extra staff (i.e. research assistants and study investigators) being able to provide operational and medical assistance at the clinic and laboratory.

**Potential for harm arising from study drugs or clinical testing**

Although anticipated to be unlikely, potential medication side effects are monitored as detailed above. Filtered mouthpieces used with both the spirometer and FeNO analyser are stated by the manufacturers to provide protection against transmission of TB. Occupational health and safety standards for staff are upheld, including safe practices in respiratory precautions and biohazard disposal.

**Disincentive due to requirements of study participants**

Additional time required to perform the extra investigations (e.g. questionnaires, exercise tolerance, lung function) might be a disincentive for some patients. Eligible patients may decline to consent if they deem that the amount of time required to fulfil
the study requirements is excessive. Research staff members ensure that the clinic environment is welcoming. A television has been installed to alleviate boredom in study participants, of particular use for those having PK / PD evaluations. RAs offer flexibility where possible (e.g. scheduling visits to suit the patient). RAs ensure that those who miss or decline some measures, or withdraw altogether, continue to receive their TB therapy.

**Feasibility of continuation after study completion**

Vitamin D and L-arginine are relatively cheap and readily available and therefore, if shown to be beneficial, would be able to be utilised as adjunctive treatments after completion of the trial at the study site, more widely in Indonesia and potentially in similar settings in other countries.

**CONFIDENTIALITY AND DATA STORAGE**

All data collected from individuals is stored confidentially on password-protected computers accessible only to the named investigators, data entry clerks and named research students. Paper records are stored in duplicate in separate filing cabinets at the NIHRD-MSHR research building in Timika. Appreciating the risk of loss of data due to computer failure, power blackout, flood or other events, data is backed up at least weekly, sent to and stored at the collaborating institutions.

**QUALITY CONTROL AND QUALITY ASSURANCE**

Study investigators evaluate and assess all aspects of the work to ensure that the study operates according to GCP guidelines, and that data collected is of as high quality as possible given the constraints of working in the resource-limited setting of the field research site.

**ELIGIBILITY CRITERIA AND INFORMED CONSENT PROCESS**

RAs receive thorough training in the processes of ensuring eligibility and obtaining informed consent. I check each enrolment form to ensure that the correct diagnostic criteria have been applied and that only eligible patients have been included. I check that all consent forms have been signed by the RA and study participant or guardian if
Chapter 6: Methods

aged <18 years. During frequent visits to the field research site, I participate at the TB clinic in enrolling and following up patients to ensure that processes are in keeping with the study protocol and with GCP requirements, including that informed consent is being obtained in the appropriate manner as set out in this protocol.

**Accuracy of data collection and data entry**

RAs’ techniques in all aspects of data collection and recording (performing accurate measurements, ensuring consistency between RAs, maintaining the equipment, transcribing results accurately to the data collection forms and writing legibly), are reviewed by me on an ongoing basis. Electronic entry of data is only performed after data collection forms have been checked for accuracy and completeness. Any apparent queries or errors are clarified with RAs. Clinical and laboratory data are double-entered by two data entry staff into an EpiData® 3.1 database, created by me for the purpose of this study (Appendix 15.12). Any discrepancies between the two records are checked against primary sources and corrected. SGRQ results are entered into a locked excel calculator provided by the questionnaire’s creators (Professor P.W. Jones, St George’s Hospital Medical School, London, UK).

**Data handling and record keeping**

The NIHRD and MSHR have co-ownership of the data, consistent with the Memorandum of Understanding (MOU) between the two institutions, under which the AVDAPT study was approved. Data is held in Timika for the duration of the trial. Back-up copies of data are regularly sent as required by the relevant ethical committees and for safe-keeping in electronic format to investigators directly involved in this research at NIHRD (Jakarta), MSHR (Darwin) and ANU (Canberra). The field data collected will be held by the principal investigators for 10 years after publication, in accordance with international best practice requirements, and then destroyed.
6.5 HEALTHY VOLUNTEER SUB-STUDY

As shown in Figure 6.1, a sub-study also approved by the NIHRD, MSHR and ANU ethics committees is being conducted alongside the AVDAPT trial, in which healthy volunteers are recruited in order to establish normal Papuan and Non-Papuan population values for serum vitamin D, amino acids and T cell CD3 zeta expression, exhaled nitric oxide levels, the 6 minute walk test and the modified St George’s Respiratory Questionnaire. Normal local reference ranges for pulmonary function have already been established at the field research site. The selected target sample size is 100 participants, comprising 50 each of healthy adult Papuans and Non-Papuans (25 males and females in each group).

SITE

Healthy volunteers are enrolled at the Timika TB clinic.

INCLUSION CRITERIA

To be eligible, healthy adults must be >18 years of age and must provide written informed consent to being enrolled. Chronic stable conditions such as hypertension which will not impact on ability to perform 6 minute walk or other tests are permitted. Smokers are included.

EXCLUSION CRITERIA

Exclusion criteria include pregnancy, hospitalisation currently or within the last month, current illness including asthma, diagnosis by a doctor of any acute illness or active infection, fever or history of fever in the last week, uncontrolled baseline hypertension: systolic blood pressure > 180mmHg, diastolic blood pressure > 100mmHg, uncontrolled baseline tachycardia, pulse > 120 bpm, or unstable angina pectoris.

ENROLMENT PROCEDURE

Healthy volunteers are sought from among friends and relatives of TB patients, after they have received TB contact tracing and are found to have no evidence of active TB,
and from local staff, friends and family members. The Research assistants seek written informed consent from potential control subjects after providing verbal and written information (see Appendix 15.13) regarding the purpose of their participation and what is required of them.

**Tests performed**

Data collection forms are reproduced in Appendix 15.14. Data collected for each healthy volunteer includes: age, sex, ethnicity, educational and employment status, smoking status, pulse and blood pressure. Consistent with the methods described earlier in this chapter, exhaled nitric oxide is measured using the portable NiOX MINO® analyser, 6 minute walk testing is performed on the standard walking track, and the modified St George’s Respiratory Questionnaire is completed.

Blood is collected for plasma vitamin D, plasma amino acids (and metabolites), and white cell function and phenotype (including T cell CD3 zeta expression), and processed in the same manner as blood collected from AVDAPT study participants. Haemoglobin is tested using a point-of-care HemoCue® analyser.

Many volunteers are also interested in having their pulmonary function tested, hence this is offered but not essential, as normal reference ranges for spirometric values have already been established in Timika.¹⁶⁵

Healthy volunteers are issued with a certificate showing their results. If any results (e.g. haemoglobin, blood pressure) are found to be abnormal, standard local management is implemented (e.g. for anaemia: diagnostic workup [blood film for malaria, investigation of potential bleeding sources] and treatment [anti-malarials if indicated according to local guidelines, iron supplementation if indicated, further referral e.g. to gynaecology clinic if required]).

Data entry and record keeping are consistent with practices described above for the AVDAPT study.
Chapter 7: Results I - Baseline

7 Results I: Baseline data in study participants and healthy volunteers

Between June 12th 2008 and October 3rd 2009, 162 participants were enrolled in the AVDAPT study. The aims of this chapter are to describe baseline characteristics of these study participants, and to compare their baseline findings with healthy volunteers.

7.1 INTRODUCTION

A key marker of TB disease severity is sputum smear grade. However, additional indicators of disease severity measured in AVDAPT study participants at baseline and serially during treatment have been selected in order to capture a broader assessment of disease severity, encompassing clinical (symptoms and weight), physiological (spirometry), functional (6 minute walk test) and quality of life (modified St George’s Respiratory Questionnaire, SGRQ) evaluations. Patient-reported quality of life outcomes are increasingly valued in the assessment of TB management and outcome, in addition to traditional clinical and biological indicators. Six minute walk testing (6MWT) and calculation of 6 minute weight · walk distance are acknowledged as useful measures of functional exercise capacity. This low-technology, essentially cost-free test is ideally suited for measuring TB-related functional impairment in under-resourced TB-endemic settings. The SGRQ is a health-related quality of life instrument formulated for use in chronic obstructive pulmonary disease, but validated in a number of respiratory diseases, including TB. Early reversible lung impairment in pulmonary TB and long-term residual impairment after TB are evaluable using spirometric measures such as forced expiratory volume in 1 second (FEV\textsubscript{1}). The initial aims of this chapter are to explore the baseline demographic characteristics of AVDAPT study participants, and to present their clinical and laboratory findings at their time of enrolment into the study.

Reference ranges in the Timika population are currently lacking for the modified St George’s Respiratory questionnaire (hereafter abbreviated SGRQ) and 6MWT. The second aim of this chapter is thus to report reference ranges for these measures among
locally recruited healthy volunteers, and to compare these with values obtained in the TB patients.

7.2 METHODS

Recruitment of consenting, eligible subjects into the AVDAPT study and associated healthy volunteer study commenced on June 12th 2008 according to the methods detailed in Chapter 6. These studies remain underway, with completion projected by early 2012. Data analysed in this thesis comprise all which had been entered into the electronic database and checked for accuracy using the duplicate data entry verification system with cross referencing to original case record forms, up to and including October 3rd 2009; this includes all study participants enrolled up to that date.

Methods of participant testing are described in Chapter 6. Participant information, consent and data collection forms are reproduced in Appendix 15.5. Additional clarification of methods is provided in the following points:

- Quality control (QC) measures in place include:
  - Continual review and cross-checking of RA’s techniques in conducting questionnaires, clinical measures and documenting results, and of laboratory technician’s blood-processing methods and results documentation.
  - Spirometer: daily check of volume reading using a standard 3L syringe.
  - Grading of acid fast bacillus (AFB) density in sputum specimens: (1) specimens collected at weeks 0, 4 and 8 are examined (either the same or simultaneously collected specimens) at both the local Timika laboratory and the University of Indonesia Faculty of Microbiology laboratory, allowing comparison of smear grading; (2) blinded QC testing is performed on a select number of stored slides by visiting trained laboratory staff.

- To be eligible for enrolment, all AVDAPT study participants require demonstration of AFB in sputum (i.e. smear positive pulmonary TB) on at least 2 sputum specimens prior to referral of the patient to the study research staff. Additional to these diagnostic specimens, a further sputum specimen is obtained
Chapter 7: Results I - Baseline

at enrolment for culture and drug susceptibility testing at the reference laboratory, and for smear grade assessment at the Timika laboratory. The result of this specimen is the one reported here; thus a small proportion of study participants have a negative enrolment sputum smear.

- Standard definitions are used, shown in Box 7.1:

**Box 7.1: Definitions**

<table>
<thead>
<tr>
<th>Nutritional status</th>
<th>Defined according to body mass index (BMI) in kg/m² (see reference(^\text{170})):</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>Pulmonary function</th>
<th>Defined according to FEV(_1) (see reference(^\text{171})):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal lung function: ≥80% predicted FEV(_1); Mild lung function impairment: 70-79% predicted; Moderate: 60–69% predicted; Mod-severe: 50–59% predicted; Severe: 35–49% predicted; Very severe: &lt;35% predicted.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Outcome</th>
<th>TB treatment outcome at 6 months (see reference(^\text{172})):</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Cured:</strong> A patient who was initially culture or sputum smear microscopy at the beginning of the treatment but who was smear-negative in the last month of treatment and on at least one previous occasion.</td>
</tr>
<tr>
<td></td>
<td><strong>Treatment completed:</strong> A patient who completed treatment but who did not meet the criteria to be classified as a cure or a treatment failure. This definition applies to pulmonary smear-positive and smear-negative patients and to patients with extrapulmonary disease.</td>
</tr>
<tr>
<td></td>
<td><strong>Treatment failure:</strong> (i) A new patient who is culture or sputum smear microscopy positive at five months or later during treatment, or who is switched to Category IV treatment because sputum culture revealed MDR-TB; (ii) A previously-treated patient who is culture or sputum smear microscopy positive at the end of the re-treatment regimen or who is switched to Category IV treatment because sputum culture revealed MDR-TB.</td>
</tr>
<tr>
<td></td>
<td><strong>Died:</strong> A patient who died from any cause during the course of treatment.</td>
</tr>
<tr>
<td></td>
<td><strong>Defaulted:</strong> A patient whose treatment was interrupted for ≥2 consecutive months.</td>
</tr>
<tr>
<td></td>
<td><strong>Transferred out:</strong> A patient who was transferred to a health facility elsewhere and for whom the treatment outcome is not known.</td>
</tr>
<tr>
<td></td>
<td><strong>Successfully treated:</strong> A patient who was cured or who completed treatment.</td>
</tr>
</tbody>
</table>
- Predicted FEV1 was calculated according to coefficients and constants previously established in the local Timika population\(^{165}\) as follows (where male=0, female=1):

  - **Papuans:** Predicted FEV1 =
    \[
    (3.9 \times \text{height in metres}) - (\text{age} \times 0.020) - (\text{gender} \times 0.551) - 2.558
    \]

  - **Non-Papuans:** Predicted FEV1 =
    \[
    (4.13 \times \text{height in metres}) - (\text{age} \times 0.010) - (\text{gender} \times 0.575) - 3.114
    \]

**Statistical analyses**

Statistical analyses were performed with Stata software (version 10.1; Statacorp), and graphs were prepared in GraphPad (version 5.0; Prism). Two-sided values of p<0.05 were considered significant. Proportions were compared between groups using Pearson’s \(\chi^2\) test, or Fisher’s exact test where appropriate. Differences in continuous data between groups were tested using 2-sample t-tests or analysis of variance (ANOVA) for normally distributed data. For non-normally distributed data, transformations were applied to achieve a normal distribution (e.g. natural logarithm), or non-parametric tests were used (Wilcoxon Rank-Sum or Kruskal-Wallis tests). Where the effect of more than one variable on continuous normally distributed data needed to be tested, multiple linear regression models were used, and residuals were examined to ensure that required assumptions were met. Weighted kappa scores (linear weighting) were used to measure inter-rater agreement for categorical variables. Kappa results were interpreted as follows: kappa<0.20: poor agreement; 0.21 - 0.40: fair; 0.41 - 0.60: moderate; 0.61 - 0.80: good; 0.81 - 1.00: very good.\(^{173}\)

7.3 Results

**Recruitment of study participants and healthy volunteers**

Four hundred and ninety two patients were commenced on TB treatment at the Timika TB clinic in the 16 months between 12/06/08 and 03/10/09 (Figure 7.1). One hundred and seventy two eligible people (smear positive, non-pregnant adults with new TB,
planning to live in Timika for the 6-month treatment duration) were referred to the AVDAPT research assistants; 8 did not consent, 2 were found to have hypercalcaemia, and 162 were enrolled. The data presented in this chapter represent 36.5% of the planned final sample size of 444 participants. Reasons for ineligibility are shown in Figure 7.1. Reasons cited for non-consent included being too busy with other duties (7), or family did not agree to the individual’s involvement (1).

Figure 7.1: Eligibility screening and enrolment of AVDAPT study participants

The proportion of females enrolled did not differ from the total proportion of females commenced on TB treatment during the recruitment period (33.3 versus 36.8%, p=0.4). However, the proportion of Papuans enrolled (48.2%) was significantly lower than the proportion starting TB treatment (59.6%) (p<0.0001, $\chi^2$), due to more Papuans being ineligible (more likely to be leaving Timika within 6 months or to be smear negative).
Forty adults comprising 20 Papuans and 20 Non-Papuans volunteered and were enrolled in the Healthy Control study. These included staff members and their colleagues, or relatives and friends of AVDAPT participants, once they had been shown on contact tracing to have no evidence of active pulmonary TB.

**Baseline data**

**Demographic and socio-economic characteristics**

Table 7.1: Baseline characteristics of AVDAPT study participants (TB patients) and healthy volunteers

<table>
<thead>
<tr>
<th></th>
<th>TB patients</th>
<th>Healthy volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of study participants</td>
<td>162</td>
<td>40</td>
</tr>
<tr>
<td>Age in years: median (range)</td>
<td>27 (15 – 65)</td>
<td>26.5 (18 – 65)</td>
</tr>
<tr>
<td>Papuan: no. (%)</td>
<td>78 (48.2)</td>
<td>20 (50.0)</td>
</tr>
<tr>
<td>Female: no. (%)</td>
<td>54 (33.3)</td>
<td>9 (22.5)</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Current or ex-smokers: no. (%)*</td>
<td>93 (57.4)</td>
<td>19 (47.5)</td>
</tr>
<tr>
<td>Never smoked: no. (%)</td>
<td>69 (42.6)</td>
<td>21 (52.5)</td>
</tr>
<tr>
<td>Highest educational attainment: no. (%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No schooling</td>
<td>14 (8.7)</td>
<td>1 (4.6)</td>
</tr>
<tr>
<td>Primary school</td>
<td>32 (19.9)</td>
<td>3 (13.6)</td>
</tr>
<tr>
<td>High school</td>
<td>112 (69.6)</td>
<td>17 (77.3)</td>
</tr>
<tr>
<td>Academy or university</td>
<td>3 (1.8)</td>
<td>1 (4.6)</td>
</tr>
<tr>
<td>Occupation: no. (%)†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Employed</td>
<td>75 (49.0)</td>
<td>25 (78.1)</td>
</tr>
<tr>
<td>Unemployed</td>
<td>66 (43.1)</td>
<td>3 (9.4)</td>
</tr>
<tr>
<td>Attending school</td>
<td>12 (7.8)</td>
<td>4 (12.5)</td>
</tr>
<tr>
<td>Owns telephone: no. (%)</td>
<td>90 (55.6)</td>
<td>24 (60.0)</td>
</tr>
</tbody>
</table>

*p= 0.07 calculated using $\chi^2$ test. †p<0.0001 calculated using $\chi^2$ test. All other differences between groups are non-significant.

Baseline characteristics of the 162 AVDAPT study participants (TB patients) and the 40 healthy controls are shown in Table 7.1. Median age was slightly lower in Papuan than Non-Papuan TB patients (25.5 vs 30.0 years respectively, p=0.01, Wilcoxon Rank Sum test). The gender ratio did not differ between ethnic groups (p=0.1; $\chi^2$ test). Males were significantly more likely to be current or ex-smokers than women (Figure 7.2). Slightly more Pauans than Non-Pauans were either current or ex-smokers (50 vs 43%) but the difference was non-significant ($\chi^2$, p=0.1).
Figure 7.2: Smoking rates in male and female AVDAPT study participants and healthy volunteers*

![Graph showing smoking rates](image)

*p values were calculated from $\chi^2$ or Fisher’s exact tests

Educational attainment did not significantly differ between men and women ($\chi^2$ test, p=0.9), but Papuans achieved significantly lower educational levels than Non-Papuans (Figure 7.3). Papuan people were also less likely than Non-Papuan people to be employed (Figure 7.4) or to own a telephone.

Figure 7.3: Educational attainment in Papuan and Non-Papuan study participants*

![Graph showing educational attainment](image)

*Papuans significantly more likely to have no or primary schooling only, p<0.0001 ($\chi^2$ test)
Figure 7.4: Employment status in Papuan and Non-Papuan study participants

![Figure 7.4: Employment status in Papuan and Non-Papuan study participants](image)

*P value calculated using $\chi^2$ test

**Clinical findings**

**Table 7.2: Symptoms**

<table>
<thead>
<tr>
<th></th>
<th>TB patients</th>
<th>Healthy volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td></td>
</tr>
<tr>
<td>Cough: no. (%)</td>
<td>162 (100)</td>
<td>-</td>
</tr>
<tr>
<td>Mild</td>
<td>14 (8.9)</td>
<td>-</td>
</tr>
<tr>
<td>Moderate</td>
<td>69 (43.7)</td>
<td>-</td>
</tr>
<tr>
<td>Severe</td>
<td>67 (42.4)</td>
<td>-</td>
</tr>
<tr>
<td>Very severe</td>
<td>8 (5.1)</td>
<td>-</td>
</tr>
<tr>
<td>Sputum: no. (%)</td>
<td>162 (100)</td>
<td>-</td>
</tr>
<tr>
<td>Haemoptysis: no. (%)</td>
<td>49 (30.4)</td>
<td>-</td>
</tr>
<tr>
<td>Diagnostic delay: median (range) months</td>
<td>2 (0.25-24)</td>
<td>-</td>
</tr>
<tr>
<td>Symptom tally: mean no. symptoms (95% CI)</td>
<td>7.7 (7.3 - 8.2)</td>
<td>-</td>
</tr>
<tr>
<td>Weight (kg): mean (95% CI) †</td>
<td>48.4 (47.3-49.6)</td>
<td>62.5 (59.2-65.8)</td>
</tr>
</tbody>
</table>

All patients reported cough and sputum production at baseline and 30.4% had haemoptysis (Table 7.2). Median reported diagnostic delay (duration of illness from onset until enrolment) was 2 months, with a positively skewed distribution; 17 patients (10.5%) reported illness duration of 1 year or more prior to being diagnosed with TB. Reported illness duration did not differ between men and women ($p=0.2$, Wilcoxon rank-sum test), but was longer in Papuans than Non-Papuans in univariate analysis; this
difference did not remain significant after controlling for educational level in a linear regression model of illness duration (log-transformed to achieve a normal distribution).

Table 7.3: Baseline clinical findings and haemoglobin

<table>
<thead>
<tr>
<th></th>
<th>TB patients</th>
<th>Healthy volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>162</td>
<td>38</td>
</tr>
<tr>
<td>Height (m): mean (95% CI)</td>
<td>1.58 (1.57-1.60)</td>
<td>1.61 (1.58-1.63)</td>
</tr>
<tr>
<td>BMI (kg/m²): mean (95% CI)</td>
<td>19.3 (18.9-19.7)</td>
<td>24.3 (23.0-25.5)</td>
</tr>
<tr>
<td>Normal BMI: no. (%)</td>
<td>101 (62.4)</td>
<td>36 (94.7)</td>
</tr>
<tr>
<td>Mild malnutrition: no. (%)</td>
<td>34 (21.0)</td>
<td>2 (5.3)</td>
</tr>
<tr>
<td>Moderate malnutrition: no. (%)</td>
<td>13 (8.0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Severe malnutrition: no. (%)</td>
<td>14 (8.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Number</td>
<td>162</td>
<td>22*</td>
</tr>
<tr>
<td>FEV1(L): mean (95% CI)</td>
<td>1.84 (1.73–1.94)</td>
<td>2.86 (2.43-3.29)</td>
</tr>
<tr>
<td>% Predicted FEV1(L): mean % (95% CI)</td>
<td>63.7 (60.6–66.7)</td>
<td>92.0 (83.2-100.8)</td>
</tr>
<tr>
<td>Lung function class: no. (%)</td>
<td>40 (24.7)</td>
<td>16 (72.7)</td>
</tr>
<tr>
<td>Normal lung function</td>
<td>21 (13.0)</td>
<td>3 (13.6)</td>
</tr>
<tr>
<td>Moderate</td>
<td>25 (15.4)</td>
<td>2 (9.1)</td>
</tr>
<tr>
<td>Mod-severe</td>
<td>27 (16.7)</td>
<td>1 (4.6)</td>
</tr>
<tr>
<td>Severe</td>
<td>38 (23.5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Very severe</td>
<td>11 (6.8)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Number</td>
<td>141</td>
<td>35</td>
</tr>
<tr>
<td>Modified St George’s Respiratory Questionnaire:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>median (range)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptoms</td>
<td>45.8 (0-90.8)</td>
<td>0 (0-21.5)</td>
</tr>
<tr>
<td>Activity</td>
<td>40.8 (0-100)</td>
<td>0 (0-12.8)</td>
</tr>
<tr>
<td>Impact</td>
<td>36.6 (0-89.0)</td>
<td>0 (0-15.1)</td>
</tr>
<tr>
<td>Total</td>
<td>40.7 (5.2-91.9)</td>
<td>0 (0-9.2)</td>
</tr>
<tr>
<td>Number</td>
<td>160</td>
<td>40</td>
</tr>
<tr>
<td>6 minute walk test (m): median (range)</td>
<td>410 (0 – 612)</td>
<td>485 (360-640)</td>
</tr>
<tr>
<td>Number</td>
<td>162</td>
<td>40</td>
</tr>
<tr>
<td>Haemoglobin (g/dL): mean (95% CI)</td>
<td>12.3 (12.0 – 12.5)</td>
<td>13.7 (12.6-14.7)</td>
</tr>
</tbody>
</table>

*FEV1* not routinely included since normal reference ranges for FEV1 already established in Timika. †P values <0.0001 calculated from 2-sample t-tests. ‡p values<0.0001 calculated using Wilcoxon rank sum tests. §p=0.0002 calculated form 2-sample t-test.

At diagnosis, study participants reported a broad range of symptoms; the average number of reported symptoms was 7.7. Most common symptoms are shown in Figure
7.5. Females reported slightly more symptoms than men (women: 8.6 symptoms, 95% CI 7.8-9.5; men: 7.3 symptoms, 95% CI 6.8-7.8; p=0.004), but did not report more severe cough than men (p=0.5, \( \chi^2 \) test).

Figure 7.5: Symptoms reported among AVDAPT study participants at TB diagnosis

Papuan males with TB had significantly higher mean BMI at baseline (20.3 kg/m\(^2\), 95% CI 18.1-19.3) than Non-Papuan males (18.7 kg/m\(^2\), 95% CI 19.8-20.9), since their weight was greater (52.3 vs 49.3 kg, p=0.0001) and height marginally lower (1.61 vs 1.62 m, p=0.3). Only 7.6% of Papuan males were classed as having malnutrition.
Chapter 7: Results I - Baseline

(BMI<18.5), compared with 52.7% of Non-Papuan males; this trend was noted among females, but did not reach statistical significance (Figure 7.7). On sub-grouping Pauans into Highland and Lowland tribes, no differences in BMI were observed between these groups.

Lung function was impaired in three quarters of study participants (Table 7.3), with mean percentage of predicted FEV$_1$ falling in the category of moderate impairment (FEV$_1$ 60-69% predicted$^{171}$). No differences in % predicted FEV$_1$ were seen between gender or ethnic groups (p>0.5, 2-sample t-tests) or according to age (p=0.2 calculated from regression of % predicted FEV$_1$ against age).

SGRQ total scores were available in 141 study participants at baseline; results were not recorded in 7 instances, and a total score could not be calculated in a further 14 people due to missing answers to parts of the questionnaire. Responses were normally distributed (but not among healthy volunteers, hence results are presented in Table 7.3 as medians), and did not differ among patient gender or ethnic groups. Results were high in each domain score and the total score. These respectively indicate presence of respiratory symptoms, restricted activities due to respiratory symptoms, perceived impact on daily living, and overall impairment in quality of life.

**Figure 7.7: Nutritional status in AVDAPT study participants**

![Nutritional Status Chart]

- **p<0.0001 calculated using $\chi^2$ test comparing nutrition group in Papuan and Non-Papuan males**
- **p=0.6 calculated using $\chi^2$ test comparing nutrition group in Papuan and Non-Papuan females**
Six minute walk distances were very limited in those patients who had significant malaise; 1 patient with copious vomiting scored 0m walk distance, and 19 of 160 participants who attempted the walk test (11.9%) walked less than 300m (Figure 7.13 illustrates 6MWT distributions). Males walked significantly further than females (median walk distances 428.0m and 373.5m respectively, p<0.0001; see Figure 7.13).

**Laboratory and radiological findings**

Consistent with expected haemoglobin (Hb) ranges, women had significantly lower mean Hb (11.5g/dL) than men (12.7 g/dL). These readings are below or at the lower limit of the standard international reference ranges for Hb of 13.5-17.5 g/dL for men and 11.5-15.5 g/dL for women (Figure 7.8). Papuan ethnicity and HIV positivity (but not age or smoking status) also significantly predicted lower Hb levels (Figure 7.8). Female sex, Papuan ethnicity and HIV positive status each remained independently predictive of lower Hb in a multivariate linear regression model (data not shown).
Table 7.4: Baseline laboratory and radiological findings

<table>
<thead>
<tr>
<th></th>
<th>TB patients*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ionised calcium (mmol/L): mean (95% CI)</td>
<td>1.21 (1.20 – 1.22)</td>
</tr>
<tr>
<td>HIV positive: no./no.tested (%)</td>
<td></td>
</tr>
<tr>
<td>Papuan†</td>
<td>18 / 138 (13.0)</td>
</tr>
<tr>
<td>Non-Papuan</td>
<td>4 / 71 (5.6)</td>
</tr>
<tr>
<td>Density of acid fast bacilli in sputum: no.(%) (Sputum smear grade)</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>10 (6.3)</td>
</tr>
<tr>
<td>Scanty</td>
<td>26 (16.4)</td>
</tr>
<tr>
<td>1+</td>
<td>49 (30.8)</td>
</tr>
<tr>
<td>2+</td>
<td>41 (25.8)</td>
</tr>
<tr>
<td>3+</td>
<td>33 (20.8)</td>
</tr>
<tr>
<td>Chest radiograph</td>
<td></td>
</tr>
<tr>
<td>Cavitation: no.(%)</td>
<td>88 (56.1)</td>
</tr>
<tr>
<td>Percentage of lung affected: median (range)</td>
<td>40 (0-100)</td>
</tr>
<tr>
<td>Score‡: median (range)</td>
<td>68 (0-140)</td>
</tr>
<tr>
<td>Sputum culture at diagnosis</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>157</td>
</tr>
<tr>
<td>No growth</td>
<td>14 (8.9)</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em> identified</td>
<td>123 (78.3)</td>
</tr>
<tr>
<td>Contaminated &amp; negative after decontamination</td>
<td>2 (1.3)</td>
</tr>
<tr>
<td>Report pending</td>
<td>18 (11.5)</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em> susceptibility</td>
<td></td>
</tr>
<tr>
<td>N</td>
<td>102</td>
</tr>
<tr>
<td>Isoniazid monoresistance</td>
<td>5 (4.9)</td>
</tr>
<tr>
<td>Streptomycin monoresistance</td>
<td>4 (3.9)</td>
</tr>
<tr>
<td>Ethambutol monoresistance</td>
<td>1 (1.0)</td>
</tr>
<tr>
<td>Isoniazid &amp; rifampicin resistance (MDR-TB)</td>
<td>2 (2.0)</td>
</tr>
<tr>
<td>Rifampicin monoresistance or other patterns of resistance combination</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

*N=162 unless otherwise stated. † p<0.01, Fisher’s exact test. ‡Chest radiograph score developed as described in Results Chapter 9.

Ionised calcium levels at baseline were independent of age, sex, ethnicity, smoking status or HIV status. HIV results are discussed in detail in Chapter 12. Chest x-ray findings are presented in Chapter 9; preliminary examination revealed no significant variation in chest x-ray scores or presence of cavitory disease according to sex, ethnicity or age (p>0.05 in each instance).
For reasons explained in the methods section of this chapter, the sputum specimen provided by a small proportion of study participants (6.3%) was AFB-negative at enrolment despite previous specimens having been positive. Sputum smear grade at diagnosis did not differ according to sex, ethnicity or smoking status (p>0.05 in each instance calculated using \( \chi^2 \) tests) or age (p=0.6 calculated from regression of log-transformed age against sputum smear grade). HIV positive people had lower smear grades (13/18 = 72.2% had smear grade ≤1) than HIV negative people (61 of 117 = 52.1% had smear grade ≤1) but the difference was not significant (p=0.1, Fisher’s exact test).

Sputum culture was reported as negative in 14 study participants at diagnosis. The 14 specimens which were culture negative were smear positive in 8 instances and smear negative in 6. One patient with no growth from sputum at week 0 had MTB isolated from his week 4 sample. Appreciating the opportunities for cultures to be falsely negative, patients with negative baseline cultures are not currently excluded from
analyses. Diagnostic culture results were unavailable in 5 study participants due to the specimens being lost in transit.

Two patients were found to have MDR-TB; both results were confirmed with Hain GenoType® MTBDRplus assay. Additionally, one case each of isoniazid and rifampicin resistance, rifampicin monoresistance, and combined isoniazid, rifampicin, ethambutol and streptomycin resistance were reported, but in each case, further evaluation including Hain testing revealed, in each case respectively: isoniazid resistance only; no resistance; mixed MTB / non-tuberculous mycobacterial growth with fully-susceptible MTB.

_Sputum microscopy quality control results_

Inter-laboratory agreement on sputum AFB grades was able to be determined where simultaneously-collected sputum samples obtained from study participants, or the same sample divided for each laboratory, were submitted to the local Timika laboratory and the Faculty of Microbiology, University of Indonesia laboratory. This routinely occurs at week 0, 4 and 8. Inter-rater agreement was moderate (weighted kappa=0.56, standard error 0.04). The paired results in 219 of 237 samples (92.5%) were within 1 grade of each other, which is generally considered to be acceptable.

Inter-rater agreement was additionally assessed by comparing results obtained from expert mycobacteriology laboratory technicians visiting the field site from the National Institute of Health Research and Development, Jakarta, and the Faculty of Microbiology, University of Indonesia. They provided an assessment of stored slides which had been made and reported at the Timika laboratory (Table 7.5). Slides assessed by the second assessor had been in storage for over 12 months in some cases, hence some deterioration of slide quality may have accounted for the lower agreement in that instance.

_Table 7.5: Agreement in sputum AFB grade_  
_Compared between visiting technicians and Timika laboratory staff_  

<table>
<thead>
<tr>
<th></th>
<th>Complete agreement with original result: no. (%)</th>
<th>Agreement within 1 grade: no. (%)</th>
<th>Weighted kappa (standard error)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assessor 1</td>
<td>13/18 (72)</td>
<td>18/18 (100)</td>
<td>0.83 (0.16)</td>
</tr>
<tr>
<td>Assessor 2</td>
<td>11/20 (55)</td>
<td>16/20 (80)</td>
<td>0.48 (0.13)</td>
</tr>
</tbody>
</table>
Comparisons between AVDAPT participants and healthy volunteers

Forty percent of the planned healthy volunteer sample size has been recruited to date. Despite relatively small numbers, preliminary comparisons are made here with the AVDAPT study participants and will be re-examined when full enrolment is completed. The group of 40 healthy volunteers had a similar ethnic and age profile as the TB patients, but fewer women (9/40 vs 54/162), although this difference was not statistically significant. Despite having similar educational backgrounds (p=0.7, $\chi^2$ test) and similar telephone ownership rates (as a marker of socio-economic status), healthy volunteers were significantly more likely to be currently employed than people with TB (Figures 7.9-7.11).

Figure 7.9: Educational attainment in healthy volunteers and TB patients*

![Educational attainment chart](chart.png)

*no significant difference in educational attainment, p=0.7 ($\chi^2$)

Figure 7.10: Telephone ownership in TB patients and healthy volunteers
Male TB patients were more likely to smoke than male healthy volunteers (83 of 108 i.e. 76.9% versus 19 of 40 i.e. 61.3%), but this difference did not achieve significance at the 0.05 level (p=0.07, χ² test) (Figure 7.2). None of the female healthy volunteers admitted to current or past smoking.

Consistent with their healthy status and in contrast with TB patients, volunteers’ mean BMI was in the normal range (Figure 7.12). No BMI difference was observed between the 2 ethnic groups, unlike that noted among the TB patients.

* Figure 7.11: Employment status in TB patients and healthy volunteers

* Figure 7.12: BMI in healthy volunteers and AVDAPT study participants
Healthy volunteers had largely normal lung function according to previously-established local reference ranges (Table 7.3), and mostly scored zero (i.e. normal lung-related quality of life) for the SGRQ total score and individual domain scores (Table 7.3 and Figure 7.14), with no difference between gender or ethnic groups (data not shown). Median distance walked in 6 minutes was significantly further for healthy volunteers (485m) than for TB study participants (410m), and further for men than women (Figure 7.13). Normal reference values in Timika for 6-minute walking testing based on the current group of 40 controls are summarised in Box 7.2.
Figure 7.14: St George’s respiratory questionnaire total score in healthy volunteers and AVDAPT study participants*

*bars represent median SGRQ results; each point represents an individual’s result. P values calculated using Wilcoxon rank sum tests.

Box 7.2: Locally-relevant reference ranges for 6-minute walk testing

<table>
<thead>
<tr>
<th>Exercise tolerance in healthy volunteers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>MALES:</strong> Mean distance in metres walked in 6 minutes: mean (± 1.96×SD):</td>
</tr>
<tr>
<td>511m (393-629)</td>
</tr>
<tr>
<td><strong>FEMALES:</strong> Mean distance in metres walked in 6 minutes: mean (± 1.96×SD):</td>
</tr>
<tr>
<td>447m (356-538)</td>
</tr>
</tbody>
</table>

Hb was significantly higher in healthy volunteers than in people with TB (Table 7.3 and Figure 7.8). The healthy volunteers’ mean Hb levels were within standard international reference ranges for men (13.9 g/dL, 95% CI 12.7-15.2) and women (12.8, 95% CI 11.0 - 14.5), but some were frankly anaemic (men: Hb range 4.9-18.6; females: Hb range 8.8-15.1). People found to have anaemia were offered malaria testing and treatment if required, further investigation as indicated, and iron supplementation, according to local treatment protocols. No healthy controls were found to have current malaria parasitaemia.

7.4 DISCUSSION
Key findings from this analysis of preliminary data from AVDAPT study participants and healthy volunteers include: over-representation of males compared with females among TB patients in Timika; socio-economic differences between Papuan and Non-Papuan TB patients; requirement for differing interpretation of BMI in Papuan and Non-Papuan people; substantial diagnostic delay, burden of disease and loss of employment in people with TB compared with healthy volunteers, and low rates of MDR-TB among cases of newly-diagnosed TB in Timika. Additionally, locally appropriate reference ranges for SGRQ and 6MWT have been established.

**Sex difference in TB rates in Timika**

Female to male ratios among all new clinic patients diagnosed with TB (181:311) and among enrolled AVDAPT study participants (54:108) were consistent with published female to male ratios of between 0.3 (South-East Asia) and 0.5 (Western Pacific region). The relative contributions to this imbalance of case-detection or notification discrepancies versus true rate differentials due to biological or exposure differences in Timika are unclear. Elsewhere, tuberculin skin test surveys showing that TB exposure rates are equal among the sexes until adolescence suggest that the greater number of contacts males have outside the household after adolescence may be relevant in increasing their TB risk. Evidence for under-reporting among females exists in some areas where the sex ratio is higher in active than passive case detection programs. Females may be more likely to have smear negative disease, with improvements in smear-positive detection rates achievable through provision of education in how to produce a good sputum sample. Higher rates of smoking (as observed in this study) and alcoholism in males may contribute to their increased pulmonary TB risk. Female gender is also identified as a risk factor for extra-pulmonary TB. Together these data suggest that females may be less likely to be exposed to TB due to social networks, more likely to develop forms of TB other than smear-positive pulmonary TB, and less likely to have TB detected and treated. These and other biological / hormonal factor have been recently reviewed elsewhere.

Data presented here contribute to the understanding of the uneven gender ratio in several ways: firstly, the recruitment data indirectly demonstrate that females were not more likely to be ineligible for recruitment into the study on the basis of smear negative disease or other factors, since the proportion of females entering the TB clinic was the
same as the proportion being enrolled in the trial. Thus although other investigators have identified females as having poorer ability to expectorate good-quality sputum production (due for instance to cultural inhibitions) and hence lower likelihood of being diagnosed with smear positive TB, these data indirectly do not support this. Secondly, females were not less likely to consent to enrolment in the study than males, suggesting that women’s engagement with health care providers at this particular clinic does not differ from men’s. Thirdly, these data do not indicate that diagnosis was delayed among females compared with males, since they did not report longer illness duration, and were not found to have more extensive disease according to baseline sputum smear grade, X-ray score, % predicted FEV\(_1\) or SGRQ scores. Females did have significantly lower haemoglobin and 6 minute walk distance than males, but these associations are physiologically expected and unlikely to be representative of more severe disease in females. Females also reported slightly more symptoms, but this is a relatively subjective measure which may be subject to gender bias, and is not necessarily a definite representation of more severe organic disease. Overall, these findings provide indirect evidence that in the Timika population, delayed diagnosis due to different health care seeking behaviour (leading to more severe baseline disease) is unlikely to be a major factor in the gender imbalance among patients with pulmonary TB in Timika. Factors including the significantly higher male smoking and employment rates reported here, with attendant risks of TB disease and exposure respectively, might contribute to the gender imbalance.

**Socio-economic data**

Timika has a rapidly-changing socio-cultural landscape characterized by an ethnically mixed population of native (Melanesian) Papuans and immigrant (Malay) Non-Papuan Indonesians. Using education, employment and telephone ownership as markers of socio-economic status, Papuan participants in the AVDAPT study were found to be more socio-economically disadvantaged according to each indicator than Non-Papuan people. Of particular relevance in this study is that the ability to contact people by telephone provides an important mechanism by which research assistants and other clinic staff can remind study participants to attend the clinic, re-schedule appointments, trace people who have defaulted, or contact people who have been transferred to other parts of Indonesia to ensure they are continuing TB treatment. Although all measures are taken to retrieve defaulting patients who are uncontactable by phone by visiting their
house and enquiring of their whereabouts from neighbours or family, lack of a telephone remains disadvantageous in health care delivery in this setting.

Documentation of employment status in this study does not distinguish inability to continue in employment due to illness from pre-existing unemployment, which are both categorised as ‘unemployed’. Educational attainment and telephone ownership were not significantly higher among the healthy volunteers, suggesting comparable socio-economic status, yet healthy people were more likely to be employed. Thus although it is possible that TB patients were unemployed prior to becoming sick, illness-related loss of employment (an indirect cost of TB) is an alternative explanation for this finding. The particularly low employment rates among Papuans compared with Non-Papuans may additionally represent pre-existing unemployment, in keeping with their lower educational attainment.

SMOKING

A high proportion (76.9%) of male AVADPT study participants were current or ex-smokers, and smokers appear to be over-represented in TB patients compared with male healthy volunteers (61.3% of whom were current or ex-smokers), consistent with the known association between smoking and increased TB risk, and suggesting smoking may be a risk factor for development of active TB in this population. Whether this difference in smoking rates between TB patients and the general population will be observed and reach statistical significance in larger sample sizes will be re-examined when recruitment has been completed. These high reported smoking rates are comparable to WHO estimates of 50-59% of adult males being current smokers in Indonesia. Females remain relatively protected due to the perceived unacceptability of female smoking among both main ethnic groups in Timika.

CLINICAL AND LABORATORY MEASURES

Body mass index

A striking difference in BMI was observed between Papuan and Non-Papuan males. Almost all Papuan males were in the normal nutritional range according to standard BMI criteria (BMI≥18.5 kg/m²), compared with only 47.3% of Non-Papuan males.
Contrastingly, lower haemoglobin levels were seen in Papuans than Non-Papuans, Papuan males did not have lesser disease severity on the various measures described, and weight loss was commonly reported by them to have preceded the diagnosis of TB. These observations indicate that standard BMI criteria may under-estimate malnutrition in Papuan males, who have classically muscular body habitus and short stature. There is an absence of literature replicating this observation in other Melanesian populations, possibly because the opportunity for direct comparisons of this kind between Asian and Melanesian populations are limited outside of Indonesia’s Papua Province. On the other hand, lower BMI cut-offs are known to be necessary in Asian people (i.e. Non-Papuans in this dataset); while the same BMI definitions of malnutrition are used, Asian-specific lower cut-offs are recommended to define overweight and obesity. Thus a two-way bias probably exaggerates the BMI difference between Papuans and Non-Papuans. Hence subsequent analyses in this thesis include weight as well as BMI, and malnutrition is not considered to be absent merely on account of BMI being >18 kg/m² in Papuan males. Any formal recommendation to revise definitions of nutritional status according to BMI in Papuan males (±females) would require a large population sample with evaluation of additional comparative markers of nutritional status.

**Lung function**

The significant burden of illness due to TB is illustrated by the high proportion of AVDAPT study participants with impaired pulmonary function, high SGRQ scores, and limited 6-minute walk distances. Three quarters of people with TB had impaired lung function according to FEV₁, and almost half (47%) had <60% of predicted FEV₁, indicating moderate-severe impairment. Given that locally-established FEV₁ reference ranges have been used to calculate % predicted FEV₁, and that lung function impairment was uncommon among the healthy volunteers despite their high smoking rates, these data indicate that TB is highly likely to be the cause of the poor lung function among these study participants.

These findings are comparable to (slightly exceeding) the extent of lung function impairment recently published from an observational study of TB patients at the same Timika research site. In this study conducted in 2003-4, 39% of people with newly-diagnosed smear positive pulmonary TB had moderately severe to severe impairment of lung function. Although improvement by 6 months was significant, 25% still had an
FEV$_1$ in the moderately-severe to severely impaired range at treatment completion. Residual pulmonary impairment after TB has also been identified in a cohort of South African TB patients, in whom 28% had residual airflow limitation and 24% had a restrictive pattern on spirometric testing at treatment completion,$^{168}$ and in patients with a recent past history of TB in the USA, in whom >50% had significantly impaired pulmonary function (including restrictive pattern, obstructive pattern &/or low vital capacity).$^{169}$ TB is acknowledged as an important cause of non-smoking related chronic obstructive pulmonary disease.$^{184}$

**St George's Respiratory Questionnaire**

The ability of the modified SGRQ to identify impaired lung-related quality of life in people with newly-diagnosed TB is evident from the high scores in AVADPT study participants compared with healthy volunteers. Testing the modified, translated questionnaire in healthy people has not previously been performed in Timika; the distribution of responses in healthy people (Figure 7.14: median score 0, range 0-9.8), confirms the appropriateness of this tool for measuring lung-related quality of life impairment in this population. Studies of the SGRQ in healthy populations are uncommon: one general population study recruited 862 Spanish adults but permitted inclusion of people with chronic airways disease (10.2% of participants); a mean score of 5.8 (range: 0 – 92.8) was reported in the youngest (39 - 49 year) age group.$^{185}$ The low SGRQ scores obtained in the healthy Timika population are expected and appropriate, given their young age and specific exclusion of those with underlying disease.

On the scale of 0 (no lung-related quality of life impairment) to 100 (severe lung-related quality of life impairment), the mean SGRQ score of 41.8 in study participants with TB represents impaired quality of life approximately equivalent to that experienced by people with moderate chronic obstructive pulmonary disease (COPD); a study in the Netherlands found that COPD patients with predicted FEV$_1$ of ~43-67% had SGRQ total scores of 35.7 – 43.4.$^{186}$ Similar to the data presented here, Maguire and colleagues in the previous study in Timika also assessed responses among TB patients to the modified Indonesian SGRQ, reporting a mean baseline score of 45.4.$^{114}$ The SGRQ has also been shown to be valid and reliable in assessing post-TB lung-related quality of life in people with TB in the USA, in whom mean scores of 23.5 were reported.$^{167}$
6 minute walk test

Exercise capacity was very limited in the most unwell patients, and the median distance walked (410m) was low compared with healthy volunteers (485m). Six-minute walk testing, chiefly used in chronic respiratory or cardiac disease, is a simple and inexpensive test of functional exercise capacity which has rarely been reported in TB. Erhabor et al (in a conference abstract) reported mean 6-minute walk distances of 502.0m and 481.7m among male and female Nigerian adults with pulmonary TB respectively. Maguire et al established the feasibility and utility of this test in Timika, reporting low six minute weight \cdot walk distance among TB study participants at diagnosis, with significant increases during 6 months of follow up. I have chosen to report 6-minute walk distance rather than six minute weight \cdot walk distance (‘six minute work’). Multiplying the distance walked by weight favours heavier people by taking account of the extra work and oxygenation required to move a heavier body. However, weight in this under-nourished patient group has different physiological ramifications for oxygen consumption compared with weight in the heavy populations in whom weight \cdot walk distance has been studied. Furthermore, work as it relates primarily to oxygenation capacity is less relevant in this patient group, in whom reasons observed for limited exercise tolerance at the time of TB diagnosis included factors such as fatigue and vomiting, not just impaired lung function.

The healthy Timika volunteers walked a substantially shorter distance than healthy people in other studies, in whom distances of 630m, and 580m in men and 500m in women have been reported in the USA and Japan respectively. Likely reasons for these differences include short stature (average height 1.61m), hot and humid ambient conditions not conducive to vigorous exercise, the walking track length, and cultural norms regarding usual walking pace. Thus the establishment of locally-relevant reference ranges for this test in men and women of both ethnic groups is particularly important, and will be published when recruitment of healthy volunteers has been completed.

**LABORATORY RESULTS**

Haemoglobin was, as expected, found to be lower in women and HIV positive people, but was additionally found to be lower in Papuans than Non-Papuans. Whether this
reflects dietary factors, frequency of malaria, TB disease severity or other factors cannot be determined from this data.

Sputum culture results are currently incomplete. False negative culture results may arise for reasons such as loss of viability during the process of transporting specimens to the laboratory (Faculty of Microbiology, University of Indonesia, Jakarta), during specimen decontamination, or loss of power/heat in the incubator. Problems in transporting specimens from Timika to Jakarta (a distance of 3500km) were identified early in the study, including unacceptably long delays and loss of some consignments; hence an alternative transport method using a direct courier was instigated. Additionally, susceptibility results are missing for a proportion of patients due to laboratory difficulties in re-culturing the organism in the antibiotic-containing tubes for susceptibility testing. These issues have been directly addressed in quality improvement exercises undertaken regularly at this WHO-accredited laboratory by senior laboratory staff from the supranational Mycobacterium reference laboratory in South Australia (Mr Richard Lumb, IMVS, South Australia).

**MDR-TB in Timika**

MDR-TB is relatively uncommon among new cases of pulmonary TB in Timika, identified in 2 of 102 patients (1.96 %). This rate has not changed in the last 5 years. Although definitions of ‘low’ and ‘high’ MDR-TB rates are not established, the 2008 report on global MDR-TB found only 14 settings where MDR prevalence among new cases was higher than 5% (up to as high as 40.8% in Azerbaijan), all in eastern Europe / Eurasia and China. Global estimates are limited by extensive under-reporting of MDR-TB due to lack of susceptibility-testing facilities. Indonesian MDR-TB rates of 2% as quoted in the 2008 WHO report are derived from the 2003-4 Timika data. Hence how the current prevalence of MDR-TB in new cases in Timika compares with other Indonesian provinces and internationally cannot be firmly established. Regardless of the uncommon nature of MDR-TB, each case creates enormous challenges in Timika, where access to second line drugs is limited to kanamycin and levofloxacin. The Indonesian National TB program’s pilot project to commence MDR-TB treatment in 100 patients only at 2 designated hospitals in East and West Java provinces, have only recently commenced.
7.5 CONCLUSION

AVDAPT study participants are largely representative of the overall population of people seeking treatment at the Timika TB clinic. They have significant disease burden at baseline according to the objective measures of body weight, lung function, exercise tolerance and low haemoglobin, and according to their low perceived quality of life. They are significantly impaired in each of these measures in comparison with local healthy volunteers. Stratification by ethnicity will be important in subsequent analyses, given the differences identified between the 2 ethnic groups in socio-economic status and BMI. Further research to clarify ethnic differences in nutritional status definitions according to BMI is warranted. The establishment of reference ranges for SGRQ and the 6-minute walk test among healthy volunteers at the study site provides essential data by which disability among AVDAPT study participants can be compared, not only at baseline, but also at treatment completion.
8 Results II: Relationships between microbiological, clinical and functional measures of TB severity

This chapter explores the relationships between the TB disease severity measures in AVDAPT study participants described in the preceding chapter.

8.1 INTRODUCTION

Sputum AFB testing provides the basis for many aspects of TB diagnosis and management. Firstly, a finding of AFB in the sputum of a person with appropriate symptoms signifies a high likelihood of pulmonary TB in those with high pre-test probability, such as in high TB-burden countries. Secondly, the public health importance of smear positive status is well established, since smear positivity confers higher infectivity. Thirdly, smear positive status provides a measure of pulmonary pathology, since bacillary load correlates with the presence of cavitary disease, as discussed in the next chapter. However, less is understood of the relationship between sputum smear grade and other measures of disease severity. The first aim of this chapter is therefore to determine whether baseline weight, FEV₁ and six-minute walk test (6MWT) are inversely related, and modified St George’s Respiratory Questionnaire (SGRQ) score, cough severity, symptom tally (number of reported symptoms) and illness duration (in months), are directly related, to sputum smear grade.

Although an association between diagnostic delay and increased TB-related morbidity is intuitive, such a relationship is not universally apparent. For instance, a recent study by Vree and colleagues failed to find an association between length of diagnostic delay and either sputum smear grade at diagnosis or mortality. Therefore the second aim of this chapter is to determine whether illness duration prior to TB diagnosis is associated with disease severity measures at baseline, and to further explore the correlations among the severity measures, which form secondary end-points of the AVDAPT study.

There is a clear association between socio-economic status and TB, although some studies suggest that associations between lower socio-economic status and pulmonary TB risk are not always apparent. Having established in the previous chapter that Papuans in this dataset are relatively socio-economically disadvantaged, the third aim of
this chapter is to investigate the relationship between socio-economic indicators and diagnostic delay or disease severity.

**8.2 METHODS**

Methods relating to recruitment and testing of AVDAPT study participants between June 12th 2008 and October 3rd 2009 are described in Chapters 6 and 7. Statistical analyses were performed using Intercooled Stata 10.1 and graphs were created in GraphPad Prism 5. Statistical tests were two-sided, with significance for p-value <0.05. Intergroup differences in means or medians were compared using 2-sample t-tests, Wilcoxon rank sum tests, analysis of variance or Kruskal-Wallis tests as appropriate. Intergroup differences in proportions were compared using Pearson’s $\chi^2$ or Fisher’s exact tests. Regression models were used to examine relationships between variables and control for potential confounders. The goodness of fit of logistic regression models was assessed using the Hosmer-Lemeshow test. For linear regression models, residuals were examined to ensure that relevant assumptions were met. For multiple comparisons, a correlation matrix was constructed with and without application of Bonferroni corrections.

**8.3 RESULTS**

Results were obtained for 162 enrolled study participants, whose baseline characteristics are described in Chapter 7.

**Sputum AFB grade versus clinical and laboratory measures**

In Chapter 7, sputum AFB grade at baseline (graded as 0, scanty, 1, 2 or 3+) was shown to have no significant relationship to sex, ethnicity, age, or smoking status. Sputum AFB grade at diagnosis was highly predictive of sputum smear status after 8 weeks’ treatment ($p<0.0001$, $\chi^2$ test): 58 of 66 (87.9%) people whose initial sample was graded $<2+$ achieved culture conversion by 8 weeks, compared with 37 of 59 (62.7%) whose initial sample was graded as 2 or 3+.

The sputum AFB grade was significantly related to radiological severity, including both presence of cavitation on X-ray ($p=0.002$, $\chi^2$ test) and overall X-ray score ($p=0.007$, Kruskal Wallis test, see Table 8.1). It was also significantly related to nutritional status.
(weight, \( p=0.006 \) or BMI, \( p=0.03 \) see Table 8.1) in univariate analysis, and in multivariate models controlling for ethnicity, sex, age, smoking, HIV and MDR-TB. The magnitude of these associations is illustrated in Figure 8.1. Although there was an inverse relationship between AFB grade and exercise tolerance (6MWT), this did not reach statistical significance. No significant associations were identified in univariate or multivariate analyses between AFB grade and illness duration, symptom tally, \( FEV_1 \), Hb or SGRQ score (Table 8.1).

**Figure 8.1:** Relationship between smear grade at TB diagnosis and CXR score and weight

(a) CXR score (\( p=0.007 \))

(b) Weight (\( p=0.006 \))

<table>
<thead>
<tr>
<th>Variable 1</th>
<th>Variable 2</th>
<th>Test</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum smear grade</td>
<td>2-month sputum smear</td>
<td>( \chi^2 )</td>
<td>( P&lt;0.0001 )</td>
</tr>
<tr>
<td>Cough severity</td>
<td>( \chi^2 )</td>
<td>( p=0.7 )</td>
<td></td>
</tr>
<tr>
<td>Chest x-ray Score</td>
<td>Kruskal-Wallis</td>
<td></td>
<td>( p=0.007 )</td>
</tr>
<tr>
<td>Presence of cavitation</td>
<td>( \chi^2 )</td>
<td></td>
<td>( p=0.002 )</td>
</tr>
</tbody>
</table>

**Table 8.1:** Association between sputum smear grade at diagnosis and other measures of disease severity

<table>
<thead>
<tr>
<th>Independent variable</th>
<th>Dependent variable</th>
<th>Coefficient</th>
<th>Significance</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum smear grade</td>
<td>Illness duration*</td>
<td>0.11</td>
<td>( p=0.2 )</td>
</tr>
<tr>
<td></td>
<td>Symptom tally</td>
<td>0.33</td>
<td>( p=0.1 )</td>
</tr>
<tr>
<td></td>
<td>Weight</td>
<td>-1.53</td>
<td>( p=0.006 )</td>
</tr>
<tr>
<td></td>
<td>Bmi</td>
<td>-0.42</td>
<td>( p=0.03 )</td>
</tr>
<tr>
<td></td>
<td>SGRQ†</td>
<td>0.16</td>
<td>( p=0.2 )</td>
</tr>
<tr>
<td></td>
<td>6MWT‡</td>
<td>-9563.5</td>
<td>( p=0.09 )</td>
</tr>
<tr>
<td></td>
<td>%predicted FEV1</td>
<td>-2.05</td>
<td>( p=0.2 )</td>
</tr>
<tr>
<td></td>
<td>Hb</td>
<td>-0.21</td>
<td>( p=0.2 )</td>
</tr>
</tbody>
</table>

Transformations required to achieve normal distribution: *logarithmic transformation; †square root transformation; ‡square transformation.
**RELATIONSHIPS AMONG OTHER VARIABLES AT BASELINE**

Relationships between the baseline clinical and laboratory measures were examined using pair-wise correlations, applying Bonferroni correction for multiple comparisons (correlation matrices, Table 8.3a and b). Any associations between these measures and demographic variables are presented in the previous chapter.

Correlations found to be significant before Bonferroni correction were investigated further in univariate linear regression models, and multivariate models controlling for age, sex, ethnicity, smoking status and HIV status. The associations listed below were found to be significant, including in the multivariate analyses. The regression coefficient and p value for each relationship is shown in Table 8.2. The magnitudes of the relationships are illustrated in Figures 8.2-8.3.

1. Longer duration of illness prior to diagnosis and treatment was associated with more symptoms, lower body weight, higher (worse) SGRQ total scores, shorter 6-minute walk distances and lower %predicted FEV1;
2. The %predicted FEV1 was associated with lower body weight and haemoglobin;
3. Higher reported number of symptoms at baseline was associated with higher (worse) SGRQ total scores and shorter 6-minute walk distances.
4. Shorter 6-minute walk distances were associated with higher (worse) SGRQ total scores.

**Table 8.2: Regression coefficients from univariate linear regression models examining associations between clinical and laboratory measures**

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Independent variable</th>
<th>Regression coefficient</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptom tally</td>
<td>Illness duration</td>
<td>0.58</td>
<td>p=0.02</td>
</tr>
<tr>
<td>Weight</td>
<td>Illness duration</td>
<td>-1.47</td>
<td>p=0.01</td>
</tr>
<tr>
<td>6MWT</td>
<td>Illness duration</td>
<td>-14957.7</td>
<td>p=0.007</td>
</tr>
<tr>
<td>SGRQ</td>
<td>Illness duration</td>
<td>0.54</td>
<td>p&lt;0.0005</td>
</tr>
<tr>
<td>% Pred FEV1</td>
<td>Illness duration</td>
<td>-6.73</td>
<td>p&lt;0.0005</td>
</tr>
<tr>
<td>% Pred FEV1</td>
<td>Weight</td>
<td>0.65</td>
<td>p=0.001</td>
</tr>
<tr>
<td>% Pred FEV1</td>
<td>Hb</td>
<td>2.86</td>
<td>p=0.001</td>
</tr>
<tr>
<td>SGRQ</td>
<td>% Pred FEV1</td>
<td>-0.28</td>
<td>p&lt;0.0005</td>
</tr>
<tr>
<td>SGRQ</td>
<td>6MWT</td>
<td>-0.01</td>
<td>p&lt;0.0005</td>
</tr>
<tr>
<td>SGRQ</td>
<td>Symptom tally</td>
<td>0.29</td>
<td>p&lt;0.0005</td>
</tr>
<tr>
<td>6 minute walk</td>
<td>Symptom tally</td>
<td>-5347.3</td>
<td>p=0.02</td>
</tr>
</tbody>
</table>

*Each association shown remained significant in multivariate models controlling for age, sex, ethnicity, smoking status and HIV status. Transformations required to achieve normal distribution include logarithmic transformation (illness duration), square root transformation (SGRQ) and square transformation (6MWT), accounting for the high regression coefficients with the latter.
Table 8.3: Correlation matrices showing correlation coefficients (top number) and p values (bottom number) for baseline clinical and laboratory measures*

(a) Without Bonferroni correction

<table>
<thead>
<tr>
<th></th>
<th>Illness duration</th>
<th>Symptom tally</th>
<th>Weight</th>
<th>SGRQ score</th>
<th>6 min walk test</th>
<th>%Pred FEV1</th>
<th>Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illness duration</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptom tally</td>
<td>0.16</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight</td>
<td>-0.20</td>
<td>0.01</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGRQ score</td>
<td>0.32</td>
<td>0.5037</td>
<td>-0.0881</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 min walk test</td>
<td>-0.21</td>
<td>-0.22</td>
<td>0.24</td>
<td>-0.48</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Pred FEV1</td>
<td>-0.34</td>
<td>-0.08</td>
<td>0.25</td>
<td>-0.34</td>
<td>0.16</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Hb</td>
<td>-0.14</td>
<td>-0.03</td>
<td>0.20</td>
<td>-0.16</td>
<td>0.23</td>
<td>0.25</td>
<td>1.0</td>
</tr>
</tbody>
</table>

(a) With Bonferroni correction

<table>
<thead>
<tr>
<th></th>
<th>Illness duration</th>
<th>Symptom tally</th>
<th>Weight</th>
<th>SGRQ score</th>
<th>6 min walk test</th>
<th>%Pred FEV1</th>
<th>Hb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Illness duration</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Symptom tally</td>
<td>0.16</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight</td>
<td>-0.20</td>
<td>0.01</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SGRQ score</td>
<td>0.32</td>
<td>0.50</td>
<td>-0.09</td>
<td>1.00</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6 min walk test</td>
<td>-0.21</td>
<td>-0.22</td>
<td>0.2384</td>
<td>-0.48</td>
<td>1.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>%Pred FEV1</td>
<td>-0.34</td>
<td>-0.08</td>
<td>0.25</td>
<td>-0.34</td>
<td>0.16</td>
<td>1.00</td>
<td></td>
</tr>
<tr>
<td>Hb</td>
<td>-0.14</td>
<td>-0.03</td>
<td>0.20</td>
<td>-0.16</td>
<td>0.23</td>
<td>0.25</td>
<td>1.0</td>
</tr>
</tbody>
</table>

*Coefficients and p values calculated using pair-wise correlation with Bonferroni correction for multiple comparisons in (b). Significant p values marked in bold.
Figure 8.2: Relationships between lung function impairment and weight, haemoglobin, illness duration and quality of life total score (SGRQ)

Lung function impairment is defined according to established criteria: Normal >80% predicted FEV1; mild impairment 70-79; moderate impairment 60-69; moderate-severe impairment 50-59; severe impairment 35-49; very severe impairment <35.171
Figure 8.3: Relationship between number of reported symptoms and illness duration and quality of life (SGRQ) score

(a) Illness duration

(b) SGRQ total score

Dots indicate median, whiskers show interquartile ranges.

A modest association between longer diagnostic delay and lower educational level was also identified (p=0.01, Figure 8.4). No other significant associations were found between the 3 socio-economic indicators (educational attainment, employment and telephone ownership) and either the length of diagnostic delay or the severity of disease.

Figure 8.4: Illness duration in relation to educational attainment

*p value calculated using a rank-sum test comparing no/primary school with high school/academy

8.4 DISCUSSION

Salient findings from this chapter include that, among participants in the AVDAPT study, sputum AFB grade was associated with baseline weight and radiological disease extent, but not other measures of disease severity; lesser educational attainment was associated with diagnostic delay, and diagnostic delay was in turn associated with higher morbidity. Disease severity measures reported here, encompassing objective
physiological and functional measures, and patient-reported subjective assessments of quality of life and symptomatology, provide a broad assessment of illness impacts, and show varying degrees of correlation with each other.

**RELATIONSHIPS BETWEEN CLINICAL AND LABORATORY MEASURES**

Although sputum AFB grade was significantly related to two widely used measures of TB severity (radiological disease extent and weight), it was not significantly associated with a range of other clinical and functional measures. While bacteriological clearance is undoubtedly of prime individual and public health importance, focussing on bacteriology alone might therefore overlook other clinically important aspects of disease severity. Bacillary grade is determined in large part by cavitation, but extensive pulmonary TB can exist in the absence of cavitation (e.g. miliary disease or widespread consolidation), and thereby have significant impacts on lung function and quality of life, yet not be reflected in higher sputum smear grades. These novel findings support the choice of a range of secondary outcome measures in the AVDAPT study. Results of a single sputum specimen have been used in this analysis, which may not be representative of the individual’s maximum baseline smear grade, but overall accuracy of results is supported by the clear association identified between week 0 and week 8 sputum smear results.

Exploration of the relationships between other baseline measures further highlights the concept that traditional methods of evaluating the TB patient via sputum smear grade and weight provide an incomplete assessment of disease severity. For example, people who were more symptomatic, including greater number of reported symptoms, poorer exercise tolerance and worse lung function, perceived their quality of life to be lower, but quality of life assessments did not correlate with bacillary burden or weight loss.

Limited 6-minute walk distances have previously been reported among people with TB. However, the current data provide new understanding of the determinants and consequences of impaired exercise capacity specifically among pulmonary TB patients. Longer illness duration prior to TB diagnosis, a greater number of symptoms, and poorer quality of life scores, were associated with decreased exercise capacity. The absence of an association between FEV1 and exercise capacity might appear unexpected. However, a similarly absent relationship between these variables has also been reported in chronic obstructive airways disease. Most importantly, this finding
confirms the observation (noted in Chapter 7) that determinants of 6-minute walk distance among people with newly-diagnosed TB include factors other than pulmonary function alone, which direct observation of study participants would suggest includes such factors as fatigue, malaise and dehydration. Other important determinants, such as a pre-existing musculoskeletal mobility disorder, are likely to impact similarly on serial walk test results, suggesting that percentage change rather than absolute values should be evaluated in longitudinal analyses. The 6-minute walk test is therefore a valuable assessment tool, being an objective functional measure which is able to identify different aspects of TB-related disease from other measures described here, and identify those who are most ill at diagnosis.

A 2004 study from Malawi demonstrated that the extent of pulmonary disease, as assessed by chest radiography, was associated with severity of malnutrition (measured using BMI and analyses of body composition) in people with pulmonary TB. However, the finding that TB patients with more evidence of malnutrition (lower body weight and haemoglobin) have worse lung function (% predicted FEV\textsubscript{1}, i.e. adjusted for sex, weight and ethnicity, using locally-derived FEV\textsubscript{1} reference ranges) as described here, has not previously been reported. The direction of this relationship cannot be established from this cross-sectional data. While progressive weight loss and worsening lung disease are both an obvious consequence of progressive TB infection, additional interactions might exist. For example, low FEV\textsubscript{1} in chronic airways disease is associated with increased metabolic rate, greater caloric requirements, and weight loss, and decreased nutritional intake is often clinically apparent as a consequence of dyspnoea; such relationships between impaired pulmonary function and nutrition may also exist in TB. Conversely, malnutrition may impair lung tissue response to pathological insults. This finding lends support to the hypothesis that nutritional interventions in TB (either specific micronutrients such as L-arginine and vitamin D, or wholefood nutritional supplementation), might have wider benefits than direct improvement of nutritional parameters alone.

**Diagnostic delay**

Diagnostic delay is multifactorial and has been extensively investigated elsewhere. Lower educational attainment was associated with delayed diagnosis in these study participants, thereby placing Papuans at greater risk of prolonged illness duration prior
to diagnosis. This information can assist in targeting future public health strategies in Timika to this at-risk group. A range of additional explanations operating at patient and health service-provider levels are likely to play roles in diagnostic delay, although investigation of these is beyond the scope of this study.

Diagnostic delay is clearly associated with greater morbidity among the study participants. Although reported illness duration is subject to substantial recall bias, associations (which remained significant in multivariate analyses) were nevertheless identified between illness duration and body weight, exercise tolerance, lung function, symptom tally and quality of life score. Although the public health importance of early diagnosis to avoid community transmission is widely appreciated, the findings reported here additionally emphasise the major importance at the level of the individual of making a timely diagnosis of TB, in order to preserve functional capacity and quality of life. While impairments in functional capacity and quality of life respond to TB treatment, post TB residual deficits in these measures are more likely in those with more advanced disease at baseline. Patients who have more advanced disease by virtue of delayed diagnosis could potentially be even more likely to benefit from adjunctive therapies which might accelerate recovery. This will be able to be investigated in future analyses of the AVDAPT trial.

8.5 CONCLUSION

Among the secondary outcomes which will be examined in the AVDAPT study are FEV₁, weight gain, cough clearance, sputum smear conversion time, exercise tolerance (6MWT) and quality of life assessment (SGRQ) (see Chapter 6). Other outcomes (composite clinical score, exhaled nitric oxide and radiological improvement) are addressed in subsequent chapters. Results presented here indicate that significant correlations exist between some but not all of these measures, indicating that they successfully capture a comprehensive range of physiological and functional aspects of pulmonary TB, rather than merely providing alternative measures of the same processes. Original findings identified in these interim analyses will be re-assessed and submitted for publication at the time of full sample size recruitment.
9 Results III: Measuring TB radiological severity

TB severity and response to treatment can be assessed on clinical, bacteriological and radiological grounds. Radiological improvement is a secondary outcome measure of the AVDAPT trial. On reviewing the literature, it became apparent that a variety of X-ray scores are in current use for grading radiological severity in adult pulmonary TB, raising the question of which method ought to be selected for use in the trial. Currently-available systems have not been validated against outcomes, and this area is lacking in standardisation. The opportunity to develop a simple x-ray scoring method arose since radiological data had been previously collected at the field research site, and a score derived from this older dataset could be validated in the current AVDAPT data.

This chapter therefore addresses Aim 5, described in Chapter 1, of developing and validating a numerical score for grading chest X-ray severity in adults with smear positive pulmonary TB, and monitoring response to therapy. This analysis is currently under review for publication. The submitted manuscript is inserted below.


<see proof next page>
A simple, valid, numerical score for grading chest x-ray severity in adult smear-positive pulmonary tuberculosis

Anna P Ralph,1,2 Muhamed Ardiân,3,4,5 Andri Wiguna,4,5 Graeme P Maguire,6 Niels G Becker,2 Glen Droogmuller,7 Michael J Wilks,8 Govert Waramori,4 Emiliana Tjitra,9 Sandjaja,9 Enny Kenagalem,3,10 Gysje J Pontororing,10 Nicholas M Anstey,1,11 Paul M Kelly1,2

ABSTRACT

Background The grading of radiological severity in clinical trials in tuberculosis (TB) remains unstandardised. The aim of this was to generate and validate a numerical score for grading chest x-ray (CXR) severity and predicting response to treatment in adults with smear-positive pulmonary TB.

Methods At a TB clinic in Papua, Indonesia, serial CXRs were performed at diagnosis, 2 and 6 months in 115 adults with smear-positive pulmonary TB. Radiographic findings predictive of 2-month sputum microscopy status were used to generate a score. The validity of the score was then assessed in a second data set of 139 comparable adults with TB, recruited 4 years later at the same site. Relationships between the CXR score and other measures of TB severity were examined.

Results The estimated proportion of lung affected and presence of cavitation, but not cavity size or other radiological findings, significantly predicted outcome and were combined to derive a score given by percentage of lung affected plus 40 if cavitation was present. As well as predicting 2-month outcome, scores were significantly associated with sputum smear grade at diagnosis (p < 0.0001), body mass index, lung function, haemoglobin, exercise tolerance and quality of life (p < 0.02 for each). In the validation data set, baseline CXR score predicted 2-month smear status significantly (p < 0.0001), and the association remained when adjusted for child TB household contact. Combining the percentage of lung affected with presence of cavitation to a single score yielded a score for grading CXR severity, which was found to be highly reproducible between patients, and predictive of outcome.

Conclusion This simple, validated method for grading CXR severity in adults with smear-positive pulmonary TB correlates with baseline clinical and microbiological severity and response to treatment, and is suitable for use in clinical trials.

INTRODUCTION

Sputum smear microscopy, and culture where available, are standardised modalities for diagnosing and monitoring treatment response in pulmonary tuberculosis (TB). Chest radiography (CXR) provides useful information regarding disease extent and progress, but there is no agreed-upon, validated system for grading the severity of CXR abnormalities in bacteriologically proven pulmonary TB. Several methods were devised for this purpose at the time of early TB treatment trials, such as those described by the Madras TB Chemotherapy Centre in 1960,1 Simon in 19662 and the National TB and Respiratory Disease Association of the USA in 1969.3 Despite this, no system has been validated in predicting outcome in more than one patient population. Recent randomised controlled trials (RCTs)4–7 and observational studies in adults with TB8–13 illustrate this lack of standardisation in the grading of radiological severity, with each of these studies utilising different non-standardised investigator-generated systems to grade CXR severity.

The same problem of non-standardised radiological reporting has been recently articulated by Dawson et al relating to TB screening, who evaluated and recommended the Chest Radiographic Reading and Reporting System14 for TB screening in HIV-positive people.15 However, this and other screening tools16–18 seek to identify the presence of latent TB infection or active disease, and are not useful for researchers wishing to accurately document severity or response to treatment in established TB.

Problems in CXR reporting arise from the heterogeneous CXR manifestations of pulmonary TB (eg, in primary vs postprimary disease, adults vs children, immunocompetent vs immunocompromised),19–21 and to inaccuracies inherent in CXR performance and interpretation,22 including limited interobserver agreement on CXR findings.23,24 Despite these shortcomings, the utility of CXR is well established in TB diagnosis and clinical monitoring.

Associations between radiological extent and other measures such as forced expiratory volume in 1 s (FEV1), age or multidrug-resistant (MDR)-TB have previously been identified,2,5,21 but a standard, simple, numerical score, validated against TB outcome, in repeated data sets, is lacking. We therefore aimed to devise a simple CXR score for use in adults with smear-positive pulmonary TB, which predicts outcome and correlates with bacteriological and clinical severity markers, for the purpose of grading severity and monitoring treatment response in the context of TB clinical trials. We then determined the utility of the score in a separate, comparable patient population.

METHODS

Study setting

The study was conducted at a community-based TB clinic in Timika, Papua Province, Indonesia. Timika has population of ~200 000 and an estimated TB incidence of 511/100 000.25
**Participants**

Adults (>15 years) diagnosed with sputum smear-positive pulmonary TB who gave written informed consent were eligible for enrolment in the study. Study participants were recruited during two time periods: 2003–2004 (the training data set) and 2008–2009 (the validation data set). The demographic, clinical and microbiological findings and outcomes in the first data set have been reported previously.

**Chest radiography**

Standard full-size posteroanterior CXR were performed at the time of TB diagnosis and 2 and 6 months thereafter, with reports provided by a clinician at the field site (first data set, PMK), second data set, APR) and, additionally for the first data set, by one of two radiologists (MJW or JD). During the first data collection period, the presence of small (1–2 mm) or large (>2 mm) nodules, patchy or confluent consolidation, cavitation, bronchial lesions or fibrosis was reported for each of three zones (upper, middle or lower zones) in each lung. The presence of effusion or lymphadenopathy was reported, the total percentage of each lung affected by any pathology was estimated, total cavity size in millimetres was recorded and the effusion volume (percentage of lung field) was estimated. To grade the percentage of affected lung, visual estimation of the extent of opacification, cavitation or other pathology as a percentage of visible lung was made; dense opacification of a zone was graded as 100% of that zone, while patchy opacification within a zone attracted scores <100% depending on the extent of opacification. Other remarks including presence of miliary disease were recorded. During the second data collection period, a simplified CXR report method was used (percentage lung affected, cavitation (0, <4 cm, ≥4 cm), effusion (0, <25%, ≥25% of hemithorax), presence of consolidation, fibrosis, nodules, miliary disease). Reporters were blinded to HIV status, bacteriological and clinical parameters and treatment outcome.

**Sputum microscopy and clinical evaluations**

Baseline sputum microscopy was performed at the onsite laboratory and repeated at the reference laboratory on samples collected at 0, 2 and 6 months, and the density of acid-fast bacilli (AFB) was graded as 1, 2 or 3+ according to standard protocols. Baseline and follow-up evaluations included: body mass index (BMI), FEV1 (spirometry performed using MLSS55C, MicroLoop, MicroMedical), haemoglobin (Hb), measured using point-of-care HemaCue or iSTAT tests, 6 min walk test (distance walked in 6 min on a straight walking track), measured according to American Thoracic Society guidelines, and St George’s Respiratory Questionnaire (SGRQ) modified to reflect local conditions and translated into Indonesian.

Standard definitions were used for nutritional category (normal, mild malnutrition, moderate malnutrition or severe malnutrition) according to BMI and for TB treatment outcome at 6 months (cured, completed, transferred, defaulted, failed or died). Impairment in FEV1 as a percentage of predicted values was calculated using previously established local reference ranges.

**Outcome measure**

The outcome measure used in this study is 2-month sputum AFB microscopy status. Two-month smear positivity has been previously shown to predict unfavourable outcomes including treatment failure and death, and determines the need for continued intensive-phase treatment versus switching to continuation-phase therapy. Although an imperfect predictor of outcome, in the absence of suitable alternatives, it remains a commonly used surrogate end point.

**Data analysis**

Statistical calculations were performed using Intercooled Stata 10.1 (StataCorp, College Station, Texas, USA); graphs were created in GraphPad Prism 5. Statistical tests were two sided, with a p value of <0.05 indicating statistical significance. Intergroup differences in means or medians were compared using two-sample t tests, Wilcoxon rank sum tests, analysis of variance or Kruskal–Wallis tests as appropriate.

Agreement between reporters in the derivation data set was tested using the concordance coefficients, rho, for continuous variables or the kappa statistic for categorical variables. Prevalence-adjusted, bias-adjusted kappa values were calculated according to the method described by Byrt et al. Kappa values were interpreted according to guidelines given by Landis and Koch (kappa ≤0.00, poor; 0.00–0.20, slight; 0.21–0.40, fair; 0.41–0.60, moderate; 0.61–0.80, substantial; 0.81–1.00, almost perfect).

The relationships between radiographic findings and clinical outcome were examined by multivariable regression analysis, using a forward stepwise approach in which any radiological variable found to be significant (p<0.05) in univariate analysis was included in the initial model. Goodness of fit of final models was assessed using the Hosmer–Lemeshow test and compared using the likelihood ratio test. The weighting for a numerical radiological score was derived from the regression coefficients. Its ability to predict outcome in the validation data set was determined using receiver operator characteristics (ROC; area under the curve (AUC)). The relationships between this score and demographic, biological and clinical variables were determined in data sets 1 and 2 using regression models using the same principles.

**Ethics**

Approval was granted by the ethics committees of the National Institute of Health Research and Development (Jakarta, Indonesia), Menzies School of Health Research (Darwin, Australia) and the Australian National University (Canberra, Australia). Written informed consent was obtained from participants in Indonesian or an appropriate Papuan language.

**RESULTS**

Characteristics of study participants in the two data collection phases are shown in table 1. All participants had smear-positive pulmonary TB (≥2 AFB smear-positive sputum samples); the result of an additional sample provided for microscopy and culture on the day of treatment commencement is reported here. This was negative in 5.7% and 7.2% of participants in the two data sets, respectively despite their previous samples being positive. Initial smear grade predicted the likelihood of smear conversion by 2 months. In the derivation data set, failure to convert to smear negative by 2 months was observed in 60.9% of patients with a baseline smear grade of 3 and in 38.7% of patients with a baseline smear grade of 3 (p=0.051). In the validation data set, failure to convert to smear negative by 2 months was observed in 48.4% of patients with a baseline smear grade of 3 and in 11.8% of patients with a baseline smear grade of 3 (p<0.001).

CXR reports were available at baseline, 2 and 6 months for 112, 76 and 76 study participants in the first data set, and 136, 93 and 76 study participants in the second data set (incomplete in the second data set as 50 of 139 had not yet completed	
Table 1  Study participant characteristics

<table>
<thead>
<tr>
<th>Demographic details</th>
<th>Derivation data set</th>
<th>Validation data set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number</td>
<td>115</td>
<td>139</td>
</tr>
<tr>
<td>Age in years: median (range)</td>
<td>30 (17–69)</td>
<td>27 (15–65)</td>
</tr>
<tr>
<td>Female gender, n (%)</td>
<td>33 (28.7)</td>
<td>48 (34.5)</td>
</tr>
<tr>
<td>Papuan ethnicity, n (%)</td>
<td>57 (48.6)</td>
<td>66 (47.5)</td>
</tr>
<tr>
<td>Smokers, n (%)</td>
<td>38 (33.0)</td>
<td>41 (29.5)</td>
</tr>
<tr>
<td>MDR-TB, n (%)</td>
<td>2 (1.7)</td>
<td>2 (1.4)</td>
</tr>
</tbody>
</table>

Baseline clinical findings

<table>
<thead>
<tr>
<th>Variable</th>
<th>Derivation data set</th>
<th>Validation data set</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI, median (range), kg/m²</td>
<td>18.6 (14.2–25.2)</td>
<td>19.0 (12.9–32.5)</td>
</tr>
<tr>
<td>Haemoglobin, median (range), g/dl</td>
<td>11.2 (6.8–18.0)</td>
<td>12.2 (7.1–16.0)</td>
</tr>
<tr>
<td>FEV₁, median (range), litres</td>
<td>1.76 (0.49–4.12)</td>
<td>1.70 (0.59–3.56)</td>
</tr>
<tr>
<td>SGRQ total score, median (range)</td>
<td>45.3 (2.5–63.5)</td>
<td>37.8 (5.2–91.9)</td>
</tr>
</tbody>
</table>

| 6 min walk distance, median (range) m | 405 (185–625) | 410 (200–612) |

Sputum AFB smear grade at diagnosis n (%)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Derivation data set</th>
<th>Validation data set</th>
</tr>
</thead>
<tbody>
<tr>
<td>0+</td>
<td>6 (5.7)</td>
<td>10 (7.2)</td>
</tr>
<tr>
<td>Scanty or 1+</td>
<td>25 (23.6)</td>
<td>65 (45.7)</td>
</tr>
<tr>
<td>2+</td>
<td>28 (26.4)</td>
<td>35 (25.2)</td>
</tr>
<tr>
<td>3+</td>
<td>47 (44.3)</td>
<td>29 (20.9)</td>
</tr>
</tbody>
</table>

2-month smear status n (%)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Derivation data set</th>
<th>Validation data set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>25 (21.8)</td>
<td>31 (22.3)</td>
</tr>
<tr>
<td>Negative</td>
<td>81 (70.4)</td>
<td>95 (68.4)</td>
</tr>
<tr>
<td>No result available</td>
<td>9 (7.8)</td>
<td>13 (9.3)</td>
</tr>
</tbody>
</table>

6-month outcome n (%)

<table>
<thead>
<tr>
<th>Grade</th>
<th>Derivation data set</th>
<th>Validation data set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cured/completed</td>
<td>88 (76.5)</td>
<td>92 (66.2)</td>
</tr>
<tr>
<td>Died</td>
<td>3 (2.6)</td>
<td>2 (1.4)</td>
</tr>
<tr>
<td>Failed</td>
<td>2 (1.7)</td>
<td>1 (0.7)</td>
</tr>
<tr>
<td>Default</td>
<td>13 (11.3)</td>
<td>8 (5.8)</td>
</tr>
<tr>
<td>Transferred</td>
<td>9 (7.8)</td>
<td>6 (4.3)</td>
</tr>
<tr>
<td>6 months not yet completed</td>
<td>0</td>
<td>30 (21.6)</td>
</tr>
</tbody>
</table>

*All study participants had at least two prior smear-positive sputum samples; some are reported as negative since this result pertains to the additional spot specimen provided at enrolment into the study (see the methods section).

AFB, acid-fast bacilli; BMI, body mass index; FEV₁, forced expiratory volume in 1 s; SGRQ, St George’s Respiratory Questionnaire.

Table 2  Chest radiograph (CXR) results

<table>
<thead>
<tr>
<th>Number of CXR available for report</th>
<th>Derivation data set</th>
<th>Validation data set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline</td>
<td>112</td>
<td>136</td>
</tr>
<tr>
<td>2 months</td>
<td>76</td>
<td>93</td>
</tr>
<tr>
<td>6 months</td>
<td>76</td>
<td>76</td>
</tr>
</tbody>
</table>

Baseline radiological findings

<table>
<thead>
<tr>
<th>Consistency</th>
<th>Derivation data set</th>
<th>Validation data set</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concordance among dichotomous variables</td>
<td>Kappa</td>
<td>Prevalence-adjusted, bias-adjusted kappa</td>
</tr>
<tr>
<td>Presence of patchy consolidation</td>
<td>0.20</td>
<td>0.70</td>
</tr>
<tr>
<td>Presence of confluent consolidation</td>
<td>0.33</td>
<td>0.31</td>
</tr>
<tr>
<td>Presence of any consolidation</td>
<td>0.24</td>
<td>0.81</td>
</tr>
<tr>
<td>Presence of small nodules</td>
<td>0.12</td>
<td>0.06</td>
</tr>
<tr>
<td>Presence of large nodules</td>
<td>0.09</td>
<td>0.09</td>
</tr>
<tr>
<td>Presence of any nodules</td>
<td>0.19</td>
<td>0.19</td>
</tr>
<tr>
<td>Presence of fibrosis</td>
<td>0.08</td>
<td>0.06</td>
</tr>
<tr>
<td>Presence of cavitation</td>
<td>0.33</td>
<td>0.37</td>
</tr>
<tr>
<td>Presence of effusion</td>
<td>-0.35</td>
<td>-0.61</td>
</tr>
<tr>
<td>Presence of lymphadenopathy</td>
<td>0.01</td>
<td>-0.09</td>
</tr>
</tbody>
</table>

had worse lung function, with a mean percentage predicted FEV₁ of 59.0 (95% CI 54.4 to 63.6) in cavitory disease versus 68.7 (95% CI 60.6 to 76.7) in non-cavitary disease (p=0.03, two-sample t test). People with cavitary disease had slightly lower BMI (18.5, 95% CI 18.0 to 19.1) compared with those with non-cavitary disease (19.2 kg/m², 95% CI 18.5 to 20.0), but this difference was not statistically significantly (mean between-group difference 0.70 kg/m², 95% CI −0.15 to 1.59). No significant associations were identified between cavitary disease and exercise tolerance (6 min walk distance), quality of life (SGRQ total or individual domain scores) or Hb.

The amount (%) of lung affected significantly predicted all clinical and laboratory variables. Specifically, greater proportions of affected lung were significantly associated with decreasing BMI category (p<0.002, Kruskal–Wallis test), lung function category

Table 3  Agreement on radiological findings, derivation data set

<table>
<thead>
<tr>
<th>Inter-rater agreement</th>
<th>Kappa</th>
<th>Prevalence-adjusted, bias-adjusted kappa</th>
<th>Interpretation of prevalence-adjusted, bias-adjusted kappa</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence of patchy consolidation</td>
<td>0.20</td>
<td>0.70</td>
<td>Substantial</td>
</tr>
<tr>
<td>Presence of confluent consolidation</td>
<td>0.33</td>
<td>0.31</td>
<td>Fair</td>
</tr>
<tr>
<td>Presence of any consolidation</td>
<td>0.24</td>
<td>0.81</td>
<td>Almost perfect</td>
</tr>
<tr>
<td>Presence of small nodules</td>
<td>0.12</td>
<td>0.06</td>
<td>Slight</td>
</tr>
<tr>
<td>Presence of large nodules</td>
<td>0.09</td>
<td>0.09</td>
<td>Slight</td>
</tr>
<tr>
<td>Presence of any nodules</td>
<td>0.19</td>
<td>0.19</td>
<td>Slight</td>
</tr>
<tr>
<td>Presence of fibrosis</td>
<td>0.08</td>
<td>0.06</td>
<td>Slight</td>
</tr>
<tr>
<td>Presence of cavitation</td>
<td>0.33</td>
<td>0.37</td>
<td>Fair</td>
</tr>
<tr>
<td>Presence of effusion</td>
<td>-0.35</td>
<td>-0.61</td>
<td>Poor</td>
</tr>
<tr>
<td>Presence of lymphadenopathy</td>
<td>0.01</td>
<td>-0.09</td>
<td>Poor</td>
</tr>
</tbody>
</table>

Concordance among continuous variables

<table>
<thead>
<tr>
<th>Variable</th>
<th>r̂ho</th>
<th>95% limits of agreement (Bland and Altman)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total amount (%) of lung affected</td>
<td>0.85</td>
<td>28.20 to 22.46 %</td>
</tr>
<tr>
<td>Extent of cavity size (mm)</td>
<td>0.69</td>
<td>56.62 to 50.66 mm</td>
</tr>
</tbody>
</table>
Table 4  Relationship between radiological and biological parameters and 2-month sputum acid-fast bacilli density in the derivation data set, showing results of univariate logistic regression analyses*.

<table>
<thead>
<tr>
<th>Radiological parameters</th>
<th>Odds ratio</th>
<th>95% CI</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Per 1% increments</td>
<td>1.30</td>
<td>0.14 to 12.18</td>
<td>0.001</td>
</tr>
<tr>
<td>Per 20% increments</td>
<td>1.90</td>
<td>1.30 to 2.68</td>
<td>0.001</td>
</tr>
<tr>
<td>Cavitary disease</td>
<td>3.26</td>
<td>1.11 to 9.56</td>
<td>0.032</td>
</tr>
<tr>
<td>Effusion</td>
<td>1.50</td>
<td>0.59 to 3.82</td>
<td>0.580</td>
</tr>
<tr>
<td>Nodules</td>
<td>1.17</td>
<td>0.41 to 3.32</td>
<td>0.773</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>2.23</td>
<td>0.86 to 5.79</td>
<td>0.097</td>
</tr>
<tr>
<td>Lymphadenopathy</td>
<td>1.43</td>
<td>0.57 to 3.56</td>
<td>0.448</td>
</tr>
</tbody>
</table>

*OR was unable to be calculated for the two instances of military disease (both had negative sputum at 2 months).

(p<0.001, Kruskal–Wallis test), 6 min walk distance (0=0.001, linear regression) and Hb in males (p=0.003, linear regression), though not in females (p=0.4). A greater proportion of lung affected on the baseline CXR was also significantly associated with SGRQ total scores (p<0.0001, linear regression) and with sputum smear grade at diagnosis (p<0.001, Kruskal–Wallis test).

To create a CXR score, cavitation and percentage of lung affected were included as independent variables in a logistic regression model for 2-month sputum smear status. The model containing both variables was significantly better (likelihood ratio test p<0.001) than the model containing cavitation alone (likelihood ratio test p=0.016) at predicting 2-month sputum smear status. Regression coefficients were 0.05167 for proportion of lung affected and 1.26151 for presence/absence of cavitation, indicating a relative weighting of 40.27 for cavitation (1.26151 + 0.05167), thereby generating an equation for the weighted score as follows:

\[
\text{CXR score } = \text{ proportion of total lung affected(%) + 40 if cavitation present}
\]

**CXR score results**

CXR score characteristics are shown in table 2 and figures 1–4. Scores did not significantly differ according to sex, ethnicity or smoking status (p>0.05, two-sample t tests), and were not significantly associated with age in univariate or multivariate analyses. Mean baseline CXR score in people with unfavourable (positive) 2-month outcomes was significantly higher (88.2; 95% CI 76.5 to 99.9) than in those with a favourable outcome (56.8; 95% CI 49.7 to 64.0), but the range of scores in each smear grade was wide (figure 1). Scores were also significantly associated with baseline microscopy grade (figure 1). CXR scores were inversely related to BMI, FEV1, Hb and 6 min walk distance, were directly related to SGRQ total score (higher SGRQ scores indicate worse quality of life) and significantly decreased over time (figures 2–4).

**Performance of score using the validation data set**

The weighted score calculated for the new data set showed similar characteristics (table 2), including a median baseline score of 69, no significant relationship with demographic factors, significant positive association with baseline smear grade (p=0.009, Kruskal–Wallis test) and the same relationships as

4 of 7

were found in the initial data set between CXR score and each of the clinical/laboratory measures (BMI, Hb, FEV1, SGRQ total score and 6 min walk distance; p<0.05 in each case).

Comparing ROC scores to predict outcome, the weighted CXR score (AUC 0.75) was significantly better at predicting 2-month smear status than the percentage lung affected alone (ROC 0.69; p=0.013, c² test; figure 5). The optimal cut-off point for weighted CXR score (value furthest from the diagonal) was 71, at which value the sensitivity for predicting a positive sputum smear status at 2 months was 80% (95% CI 61.4 to 92.3) and specificity 67.7% (95% CI 57.3 to 77.1). Comparative sensitivity and specificity values are shown in table 5.

DISCUSSION
The current need for a universal and standard system for reporting CXR in pulmonary TB is acknowledged. In order to grade CXR severity and assess radiological treatment response, we have derived a simple equation from radiographic parameters from adults with smear-positive pulmonary TB that predicts smear positivity at 2 months and provides a single numerical score for each CXR. The score shows good correlation with baseline bacteriological and clinical severity markers, and is sensitive to changes over time. The score performs better than its individual components: it was significantly better at predicting outcome than was the percentage of lung affected alone, and was significantly associated with a broader range of baseline severity measures (BMI, Hb, exercise tolerance and quality of life) than presence of cavitation alone. Advantages of this method are that CXR assessment does not require aids, grids or rulers, and it is derived by fitting a statistical model to outcome data rather than by assigning points based on assumed relative importance of radiographic pathologies. It has been

Table 5  Sensitivity and specificity of baseline radiological and bacteriological findings in predicting outcome (2-month sputum acid-fast bacilli (AFB) status)

<table>
<thead>
<tr>
<th>Radiological parameters at diagnosis</th>
<th>Sensitivity for predicting positive 2 month smear</th>
<th>Specificity for predicting positive 2 month smear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Derivation data set</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total amount of lung affected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20%</td>
<td>96</td>
<td>35.9</td>
</tr>
<tr>
<td>37.5% (optimal cut-off point)</td>
<td>84</td>
<td>68.0</td>
</tr>
<tr>
<td>80%</td>
<td>20</td>
<td>89.7</td>
</tr>
<tr>
<td>Cavitation</td>
<td>80</td>
<td>44.9</td>
</tr>
<tr>
<td>Score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20%</td>
<td>100</td>
<td>18.0</td>
</tr>
<tr>
<td>72.5% (optimal cut-off point)</td>
<td>80</td>
<td>71.2</td>
</tr>
<tr>
<td>130%</td>
<td>8</td>
<td>96.7</td>
</tr>
<tr>
<td>Validation data set</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total amount of lung affected</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20%</td>
<td>96.7</td>
<td>22.6</td>
</tr>
<tr>
<td>42.0% (optimal cut-off point)</td>
<td>73.3</td>
<td>62.4</td>
</tr>
<tr>
<td>80%</td>
<td>30</td>
<td>87.1</td>
</tr>
<tr>
<td>Cavitation</td>
<td>83.3</td>
<td>52.7</td>
</tr>
<tr>
<td>Score</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20%</td>
<td>96.7</td>
<td>22.6</td>
</tr>
<tr>
<td>71% (optimal cut-off point)</td>
<td>80</td>
<td>67.7</td>
</tr>
<tr>
<td>130%</td>
<td>10</td>
<td>91.4</td>
</tr>
<tr>
<td>Bacteriological parameters: baseline smear grade</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Derivation data set</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFB density*</td>
<td>60.9</td>
<td>61.3</td>
</tr>
<tr>
<td>Derivation data set</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AFB density*</td>
<td>48.4</td>
<td>88.2</td>
</tr>
</tbody>
</table>

*Baseline AFB density (smear grade) dichotomised as ≥2 or 3.
Cavitation is well recognised to correlate with bacillary load. We confirmed the association between cavitation and bacteriological measures (baseline and 2-month sputum smear status), and additionally showed cavitory disease to be predictive of worse lung function. The proportion of lung affected was associated with both bacteriological and a range of clinical measures.

This score was derived in adult patients with TB with smear-positive pulmonary disease, in a setting with relatively low rates of HIV—TB co-infection and MDR-TB. The score requires further evaluation in populations with high HIV prevalence, in whom CXR findings characteristic of HIV—TB co-infection (subtle or absent pathology, non-cavitary disease, lower lobe infiltrates, hilar lymphadenopathy and pleural effusion) may mean that a differently weighted score is needed. Nevertheless, the score remained valid and applicable in the newer data set in which HIV—TB co-infection rates were higher (15%); the rise in HIV prevalence may account for some of the differences observed between the two data sets. The presence of MDR-TB would not be expected to alter radiographic patterns, other than being associated potentially with higher scores and smaller incremental improvements over time.

Potential limitations of the study include the use of 2-month smear status as an outcome measure (rather than a longer term measure such as 6-month outcome or recurrence).

The absence of suitable biomarkers or other surrogate endpoints in TB research is readily acknowledged, and recent estimates derived from meta-analysis found a sensitivity of only 57% and specificity of 81% for 2-month smear status in predicting treatment failure. Nevertheless, until more suitable measures become available, 2-month smear status remains a suitable outcome measure.

Another limitation was the inherent problem of limited inter-rater agreement in CXR assessment. The low rates of clinician—radiologist agreement between reporters on CXR findings identified in the derivation data set are not unusual, with only fair or poor agreement between radiologists and clinicians also being reported elsewhere. This emphasises the importance of using simple rather than complex scores and ensuring individuals allocating CXR scores participate in continuing education to maximise agreement. The score derived from radiologist CXR evaluation in the first data set is simple. Moreover, it was shown to be valid in the second data set when used by an independent TB clinician, rather than a radiologist, confirming its practical utility in a clinical and trial setting. Some systematic differences in CXR results were noted between the two data sets; while this may represent systematic difference in reporting styles, the findings are in keeping with the possibility of less severe disease in the validation data set, as indicated by their lower bacillary burden (with slides read by the same senior laboratory technician during both data collection periods).

CONCLUSION

In summary, we have derived and validated a simple method for grading CXR severity in adults with smear-positive pulmonary TB that predicts baseline clinical and microbiological severity and response to treatment in two separate patient populations. Although finer discriminatory accuracy might be achieved by collecting more detailed CXR findings (such as cavity size), our data did not indicate this. This method can be used where a numerical score is required for the purpose of comparing radiographic severity between adults with smear-positive pulmonary TB, and to monitor an individual’s improvement over time, such as in clinical trials of drug efficacy in TB.

REFERENCES

Acknowledgements We thank the following for their support and assistance: Dr M Okoserey, Pak Penias and Pak E Meokbin and the Timika District Health Authority; Dr Dina Bisa Lolang and Ibu Meryani Ginsang and the National Institute of Health Research and Development, Jakarta; Dr P Penfinten, Dr M Bangs and Dr M Stone, Public Health & Malaria Control (PHMC) and International SIDS; Pak Isantaro and PHMC laboratory staff; Pak J Lempoy and Timika TB clinic staff; Dr P. Sugianto and Mirmika Community Hospital (RSMMA); Natalia Dwi Haryanti, Sri Hasmunik, Sri Rahayu, G Bellatrix and clinical and laboratory staff, NIHIRD-MSHR Timika research programme; and Mr R Lamb and Dr I Bastian at the Institute of Medical and Veterinary Science.

Funding Australian Respiratory Council, the Royal Australasian College of Physicians (Covance award), Australian National Health and Medical Research Council.

Competing interests None.

Ethics approval This study was conducted with the approval of the Human Research Ethics Committees of the NT Department of Health & Families and Menzies School of Health Research, Australia, the Australian National University, and the National Institute for Health Research and Development, Indonesia.

Provenance and peer review Not commissioned; externally peer reviewed.

...
Chapter 10: Results IV – Longitudinal

10 Results IV: Longitudinal follow up

This chapter reports on the progress over time of enrolled AVDAPT study participants, including treatment outcome in those who have completed 6 months, adverse events, interim safety analysis and the development and evaluation of a composite clinical TB outcome score (a primary outcome measure of the AVDAPT study).

10.1 INTRODUCTION

Progress of individuals enrolled in the AVDAPT study can be assessed according to standard DOTS program objectives, and also according to the additional parameters of interest for the clinical trial. Adverse events and serious adverse events among study participants have been documented and reported in keeping with Good Clinical Practice (GCP). Particular attention is required regarding the possibility of increased risk of hypercalcaemia among participants randomised to vitamin D-containing arms of the AVDAPT study, or gastrointestinal disturbance in those randomised to arginine-containing study arms. An interim safety analysis was performed by an independent statistician when a quarter of study participants had been enrolled and followed up for 2 months. The two stated primary endpoints of the AVDAPT study are listed in Box 10.1.

The aims of this chapter are two-fold:

1. To document treatment outcomes among study participants, including sputum smear and culture conversion, and provide interim adverse event and safety data.
2. Describe the development of the composite clinical outcome score (the second primary endpoint), and its performance at 2 months in relation to other markers of improvement. As study arm allocation remains concealed until study completion, the current analysis does not include comparison of this endpoint among study arms.


Box 10.1: AVDAPT study primary outcome measures

(1) Proportion of pulmonary TB patients who are culture negative at 1 month
(2) Difference in improvement in composite clinical endpoint comprising weight, cough clearance and FEV\textsubscript{1} at 2 months.

The composite clinical endpoint comprises a **nutrition component** (% weight change) and a **respiratory function component** (% change in FEV\textsubscript{1}, and progress in cough, sputum and haemoptysis). Percentage weight gain is widely recognised as a valuable measure of response to TB treatment.\textsuperscript{201, 202} The long-established two-way relationship between malnutrition and increased susceptibility to infection\textsuperscript{203} is of particular importance in TB. Underweight individuals carry a higher TB risk\textsuperscript{202, 204} and TB infection causes cachexia.\textsuperscript{205, 206} Inadequate weight gain during treatment is associated with higher TB relapse risk,\textsuperscript{201} and low body mass index is associated with an increased risk of early death.\textsuperscript{207} A recent study reports that a nutritional risk score comprising BMI<18.5 kg/m\textsuperscript{2}, hypoalbuminaemia, hypocholesterolaemia and severe lymphopaenia, was predictive of mortality in hospitalised TB patients.\textsuperscript{208} TB-HIV co-infected people are even more likely to be underweight\textsuperscript{209} or have nutritional deficiencies\textsuperscript{210} than HIV negative TB patients.

Impaired lung function due to TB can be evaluated using a variety of means, with FEV\textsubscript{1} a frequently-used measure. Both early reversible lung impairment in pulmonary TB\textsuperscript{114, 168} and long-term residual impairment post-TB\textsuperscript{169} have been characterised using FEV\textsubscript{1}, described in Chapter 7.

Weight and FEV\textsubscript{1} change are therefore appropriate measures to include in an outcome score, and can be objectively assessed. Addition of clinical symptomatology (cough, sputum production, haemoptysis), routinely used in clinical practice to gauge response to therapy, to an outcome score, could improve the sensitivity of the score to clinically-relevant improvements over time. Eight and 24 weeks of follow up are standard assessment points at which to calculate the score, the former being the usual transition timepoint between intensive-phase and continuation phase therapy, and the latter representing end of standard therapy.

Other investigators have generated scores for the purpose of evaluating TB response to therapy: Wejse and colleagues constructed a score using respiratory symptoms, night
sweats, pale conjunctivae, tachycardia, abnormal chest auscultation, temperature >37, body mass index and middle upper arm circumference.\textsuperscript{211} They showed that higher absolute scores at baseline and 8 months were predictive of mortality. We however aim to use a score incorporating relative change over time, which is likely to offer greater discriminatory ability in assessing degrees of treatment response, and to use fewer, physiologically well-justified score components. In this chapter, the composite clinical outcome score we have devised for use in the AVDAPT study is calculated in two ways, generating either an ordinal score of 0 to 12, or a dichotomous score. The performance of these scores and the relationships between them and percentage change in other parameters measured during follow up is examined.

\section*{10.2 METHODS}

Sputum processing methods are described in Chapter 6 (Methods). In brief, sputum AFB smears were examined using ZN staining (Timika laboratory) and culture was performed using MGIT and Lowenstein-Jensen medium (at the FKUI laboratory, Jakarta). Sputum AFB results reported here are those obtained at the Timika laboratory unless otherwise stated.

Interim analysis was performed as scheduled when 111 study participants (25\% of the sample size) had 8-week data available. This was undertaken by the statistician member of the DSMC (Mr Joseph McDonnell). Study arms to which the participants had been randomised were identified to the statistician as Groups A, B, C and D, but concealment was maintained regarding whether active or placebo medications had been administered, and study investigators including myself were not given access to the group allocations. Safety (any differences in adverse event rates in the study arms) and balance of baseline characteristics among participants in the different study arms were assessed. Safety measures included potential adverse effects of vitamin D or L-arginine i.e. hypercalcaemia, neurological symptoms including headache, dizziness and confusion which could be attributable to hypercalcaemia, gastrointestinal symptoms, and any new respiratory symptoms suggestive of illness exacerbation. The findings of the analysis were disseminated to other members of the DSMC to assess the safety of continuation of the trial.
The composite clinical outcome score (Table 10.1) was devised *pre hoc* by consensus opinion of a panel of clinicians with collective experience in managing TB and in devising clinical trial outcome measures (Associate Professors Graeme Maguire, Paul Kelly, Peter Morris, Professor Nicholas Anstey, Dr Anna Ralph). We weighted the score components consistent with their recognised clinical importance in TB. Thus highest scores were assigned to weight gain, and the selected cut-off of weight gain <5% was guided by previously-published results. The lung function cut-offs were guided by knowledge of test repeatability (accuracy) and plausible improvements in FEV$_1$ during the given time frame. In developing the score, absolute values versus percentage changes were considered. The latter was chosen on the basis of percentage change being more able to accurately reflect an individual’s response to treatment. The resulting score is ordinal, with possible results of 0 to 12, or can be summarised as a dichotomous score.

**Table 10.1: Composite clinical outcome score**

(a) Ordinal score

<table>
<thead>
<tr>
<th>Clinical parameter</th>
<th>Outcome</th>
<th>Points</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Weight parameter</strong></td>
<td>% Weight change</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Decrease</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&lt;5% weight gain</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>5.0-9.9% weight gain</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>≥10% weight gain</td>
<td>6</td>
</tr>
<tr>
<td><strong>Respiratory parameters</strong></td>
<td>% FEV1 change</td>
<td></td>
</tr>
<tr>
<td></td>
<td>≥10% fall in FEV1</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>&lt;10% fall or &lt;10% improvement in FEV1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>≥10% FEV1 improvement</td>
<td>2</td>
</tr>
<tr>
<td><strong>Cough</strong></td>
<td>Worse or same</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Improved</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ceased</td>
<td>2</td>
</tr>
<tr>
<td><strong>Sputum</strong></td>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>1</td>
</tr>
<tr>
<td><strong>Haemoptysis</strong></td>
<td>Present</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Absent</td>
<td>1</td>
</tr>
</tbody>
</table>

**Total maximum score** 12
a) Dichotomous score: Score 1 if the weight target plus at least one of the respiratory targets are met (i.e. score 0 if weight target not met).

<table>
<thead>
<tr>
<th>Weight parameter</th>
<th>≥10% weight gain achieved</th>
<th>No / Yes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Respiratory parameters</td>
<td>≥10% FEV1 improvement achieved</td>
<td>No / Yes</td>
</tr>
<tr>
<td></td>
<td>OR</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Improvement in cough and resolution of sputum / haemoptysis achieved</td>
<td>No / Yes</td>
</tr>
</tbody>
</table>

Possible scores 0 or 1

Adverse events in the AVDAPT study are defined and reported in keeping with GCP guidelines (non-serious adverse events [AE]: any new symptom or complaint arising during the course of the study which was not present at baseline; serious adverse events [SAE]: death, life-threatening illness or hospitalisation). All SAE are reported to the Data and Safety Monitoring committee members, who assess the case and determine the relatedness of the SAE to the study medications (although because allocation concealment is maintained, the study participant may not have received active study medications).

Statistical analyses were performed in Stata 10.1 as described in previous chapters. Additionally, Kaplan-Meier survival analysis was used to examine time-to-event data (time to sputum smear conversion using weekly sputum smear readings). Patient subgroup analysis was performed by Cox regression (proportional-hazards) models and hazard ratios and 95% confidence intervals. Log-log plots were used to establish whether proportional-hazards assumptions were met.

To account for repeated conversions / reversions in smear status during the course of treatment, smear negativity was defined as the time point (week after treatment) at which the first of 3 consecutive negative results was recorded, or the first negative result if fewer than 2 subsequent results were recorded, as described elsewhere.²⁴², ²⁵⁰ McNemar’s $\chi^2$ test was used to compare proportions for paired data. Wilcoxon’s signed rank test for matched pairs was used to compare values for paired non-parametric data. For analysing associations of the composite clinical score, assumptions required for linear regression analyses could not be met, hence these were investigated using Spearman’s rank correlations.
10.3 RESULTS

Of 162 participants enrolled since June 12th 2008, 155 were enrolled at least 4 weeks, and 139 at least 8 weeks, prior to database closure on October 3rd 2009. Findings reported below are confined to those with 8-week follow up data unless otherwise indicated.

Figure 10.1: Participant follow up profile
Default = defaulted from TB treatment and study altogether. Withdrew from study = withdrew from study but continued TB treatment.

STUDY PARTICIPANTS LOST TO FOLLOW UP

Losses to follow up comprised 9 study participants who defaulted from TB treatment altogether, withdrew from the study but continued TB treatment, or were transferred prior to their week 4 appointment (9/155 = 5.8% loss to follow up by 1 month), and a further 2 who withdrew or died prior to their week 8 appointment (11/139 = 7.9% loss to follow up by 2 months). See Figure 10.1. These 11 study participants who were lost to follow up did not differ in age, sex, ethnicity, HIV, education or employment status.
from the rest of the study population (p>0.05 in each instance). Some differences were noted in baseline disease severity indicating the possibility of more severe disease according to some parameters, but none reached statistical significance. Specifically, the 11 people lost to follow up had higher (worse) baseline chest x-ray scores (median score 100 vs 68) and lower percentage of predicted FEV₁ (median % predicted FEV₁ 49.4% vs 61.5%) than other study participants (p=0.1 and 0.2 respectively). There were no statistically significant differences in baseline sputum smear grade, 6-minute walk distance, quality of life score, illness duration or symptom tally (using Fisher’s exact test or Wilcoxon rank sum tests).

**BACTERIOLOGICAL RESULTS**

**SPUTUM SMEAR AND CULTURE CONVERSION**

Of 139 study participants enrolled at least 8 weeks prior to database closure, sputum smear conversion (according to the Timika laboratory sputum microscopy result) was achieved in 60.9% and 75.6% by 4 and 8 weeks respectively, and culture conversion (according to the FKUI laboratory culture results) in 57.9% and 80.4% by 4 and 8 weeks respectively (Table 10.3 and Figure 10.2).

Reasons for culture results being unavailable included: contamination of culture and inability to successfully de-contaminate; samples not collected or lost in transit; inadequate sample present in the specimen jar; electricity failure at the laboratory resulting in incubator failure, or departure of the patient from the study (default, withdrawal from study, transfer or death).
Table 10.2: Sputum MTB microscopy and culture results at baseline and 4 and 8 weeks

<table>
<thead>
<tr>
<th></th>
<th>Baseline</th>
<th>Week 4</th>
<th>Week 8</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SPUTUM AFB MICROSCOPY</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microscopy result available</td>
<td>139/139 (100)</td>
<td>128/139 (92.1)</td>
<td>127/139 (91.4)</td>
</tr>
<tr>
<td>Smear positive: no. (%)</td>
<td>119/139 (85.6)</td>
<td>50/128 (39.1)</td>
<td>31/127 (24.4)</td>
</tr>
<tr>
<td>Reasons for microscopy unavailability</td>
<td>-</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Patient left study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient did not attend</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Patient unable to expectorate</td>
<td>-</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>SPUTUM CULTURE</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture result available</td>
<td>123/139 (88.5)</td>
<td>107/139 (77.0)</td>
<td>92/139 (66.2)</td>
</tr>
<tr>
<td>Culture Positive: no. (%)</td>
<td>111/123 (90.2)</td>
<td>45/107 (42.1)</td>
<td>18/92 (19.6)</td>
</tr>
<tr>
<td>Reasons for culture unavailability</td>
<td>-</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>Patient left study</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture contamination</td>
<td>4</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>Electricity failure</td>
<td>1</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Sample inadequate or missing from consignment</td>
<td>2</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Sample lost in transit</td>
<td>9</td>
<td>14</td>
<td>22</td>
</tr>
<tr>
<td>Patient unable to expectorate</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Figure 10.2: Microscopy and culture results at enrolment, and 4 and 8 weeks in participants followed for 8 weeks
Twenty six of 92 study participants (28%) were still smear positive at 8 weeks, and of these, 12/26 (46%) were also culture positive, the remaining 14 (54%) were culture negative. Of the 66/92 (72%) participants who were smear negative at 8 weeks, 6/66 (9%) were culture positive (Figure 10.2).

Predictors of sputum AFB smear conversion

The median time to smear conversion was 4.5 weeks (range 1 to 28). Smear conversion times did not differ according to sex, age, ethnicity or smoking status (p>0.5 in each instance, Cox proportional hazards methods). Sputum smear conversions / reversions were common during individual’s courses of treatment, providing justification for defining smear negativity as the first of 3 consecutive negative samples (as per Methods). Time to smear conversion over the 24 weeks of standard treatment is shown in Figure 10.3. Subsequent figures truncate follow up time at 8-10 weeks. HIV status did not significantly influence time to conversion; in fact conversion appeared initially more rapid in HIV positive people (Figure 10.4), but not significantly so (hazards ratio [HR] 0.7, 95% CI 0.41 - 1.3, p=0.2).

The presence of cavities on the baseline chest radiograph, and higher baseline AFB grade, were both associated with significantly slower time to sputum smear conversion (HR 0.6, 95% CI 0.4-0.96, p=0.03 and HR 0.6, 95% CI 0.5- 0.8, p<0.0001 respectively; Figures 10.5 and 10.6). The presence of isoniazid mono-resistance (identified in 5/139 study participants) did not significantly predict to time to smear conversion (HR 1.6, 95% CI 0.6-4.5, p=0.3). The impact of MDR-TB could not be modelled due to low numbers in this subgroup (1 MDR-TB patient experienced sputum smear conversion at week 11, the other remains smear positive to date).

In the following figures, the number at risk is indicated below each graph. Analyses are restricted to participants followed up for the indicated number of weeks. Number tallies are not the same in each graph since <100% of study participants were smear positive at baseline, or had known HIV status or documented CXR results.
Figure 10.3: Kaplan-Meier survival curve showing probability of sputum smear conversion by ethnicity

Figure 10.4: Kaplan-Meier survival curve showing probability of sputum smear conversion by HIV status
Figure 10.5: Kaplan-Meier survival curve showing probability of sputum smear conversion by cavitary disease status

Hazards ratio 1.6, p=0.03

Figure 10.6: Kaplan-Meier survival curve showing probability of sputum smear conversion by baseline sputum AFB grade

Hazards ratio 1.6, p<0.0001

**Predictors of sputum culture conversion**

In univariate logistic regression analyses, higher sputum smear grade at diagnosis predicted a failure to achieve sputum culture conversion at 4 and 8 weeks, while presence of cavitary disease and lower BMI (but not weight) at baseline predicted a
failure to achieve sputum culture conversion at 4 weeks. In multivariate logistic regression analyses, the association between BMI and 4-week culture conversion became statistically non-significant (p=0.1), leaving baseline smear grade the only predictor of culture conversion. No associations were identified between culture results and sex, age, ethnicity, smoking status, HIV status, baseline weight or baseline pulmonary function. Significant results in univariate analyses are shown in Table 10.4.

**Table 10.3: Predictors of sputum culture conversion, univariate analyses**

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Independent variable</th>
<th>Odds ratio*</th>
<th>95% Confidence interval</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture week 4</td>
<td>BMI at baseline</td>
<td>0.8</td>
<td>0.7 – 0.99</td>
<td>p=0.04</td>
</tr>
<tr>
<td>Culture week 4</td>
<td>Cavitary disease</td>
<td>4.4</td>
<td>1.9 – 10.4</td>
<td>p=0.001</td>
</tr>
<tr>
<td>Culture week 4</td>
<td>Baseline AFB grade</td>
<td>2.2</td>
<td>1.5 – 3.4</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Culture week 8</td>
<td>Baseline AFB grade</td>
<td>1.9</td>
<td>1.1 – 3.2</td>
<td>p=0.02</td>
</tr>
</tbody>
</table>

*Odds ratios calculated from univariate logistic regression models

**TREATMENT OUTCOME**

Six-month treatment outcomes were assessed according to the standard WHO categories (Table 10.5) and grouped as Treatment Success (cured or completed) or Unfavourable outcome (failed, died or defaulted). Treatment success was achieved in 84.5% overall, almost reaching the 85% WHO target for completion or cure.

**Table 10.4: Six-month treatment outcome**

<table>
<thead>
<tr>
<th>6-month treatment outcome: no. (%)</th>
<th>Study participants: no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>110</td>
</tr>
<tr>
<td>Cured</td>
<td>86 (78.2)</td>
</tr>
<tr>
<td>Completed</td>
<td>7 (6.4)</td>
</tr>
<tr>
<td>Failed</td>
<td>1 (0.9)</td>
</tr>
<tr>
<td>Default</td>
<td>8 (7.3)</td>
</tr>
<tr>
<td>Died</td>
<td>2 (1.8)</td>
</tr>
<tr>
<td>Transferred</td>
<td>6 (5.5)</td>
</tr>
</tbody>
</table>

Two deaths and one treatment failure occurred. Longer diagnostic delay appeared to be associated with death or failure: the illness duration prior to TB diagnosis in the 2 patients who died and 1 who failed treatment were 3, 12 and 24 months respectively,
significantly higher than the median illness duration (2 months) in those who achieved treatment success (p=0.02, Wilcoxon rank sum test). Overall, differences between outcome groups were difficult to ascertain with confidence due to the small numbers in categories other than the successful treatment group. Outcome was not found to differ according to age, gender, ethnic group, smoking status or HIV status (p>0.05 in each instance calculated from Fisher’s exact tests). Furthermore, outcome was not predicted by baseline sputum smear grade, baseline weight or BMI, or baseline X-ray score (p>0.05 in each instance calculated from Kruskal-Wallis tests or one-way ANOVA).

Unfavourable outcomes according to WHO criteria are not identical to losses to follow up for the purposes of the AVDAPT study, since firstly only 110 participants who have completed the study were examined regarding final outcome, but losses to follow up reported above includes all who had been enrolled in the study for at least 8 weeks. Secondly, the final outcome was able to be determined (cured in both instances) in 2 of the patients lost to follow up who dropped out of the study but remained under DOTS.

**Composite clinical outcome scores**

**Twelve-point score**

Composite clinical outcome scores using the 12-point scale were able to be calculated in patients who had data recorded for each score component at baseline and the comparative time point at 8 or 24 weeks. Thus scores were available in 125 of 139 study participants who had completed 8 weeks, and 81 of 110 who had completed 24 weeks (78 matched pairs). Scores increased significantly between week 8 and 24 (Table 10.6 and Figure 10.7). Higher scores indicate greater improvement.

<table>
<thead>
<tr>
<th>Composite clinical outcome score</th>
<th>8 weeks</th>
<th>24 weeks</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ordinal score: median (range)</td>
<td>6 (1-11)</td>
<td>9 (3-11)</td>
<td>p&lt;0.0001*</td>
</tr>
<tr>
<td>Dichotomous score positive (successful): no. (%)</td>
<td>18/127 (14.2)</td>
<td>38/88 (43.2)</td>
<td>p&lt;0.0001†</td>
</tr>
</tbody>
</table>

*p value calculated using Wilcoxon signed rank test for matched pairs. †p value calculated using McNemar χ² test
At both 8 and 24 weeks, composite clinical scores correlated weakly but significantly with percentage changes in SGRQ score, 6 minute walk distance and haemoglobin, using Spearman’s rank correlation (Table 10.7). (Note: a positive value for % change in SGRQ score signifies deterioration, accounting for the negative Rho values in the table below.) Median composite clinical scores at 8 weeks did not differ according to sputum microscopy and culture status at week 8 (p=0.8 and 0.9 respectively; Kruskal-Wallis tests).

**Figure 10.7: Histograms demonstrating distributions of composite clinical outcome scores at 8 and 24 weeks**

![Histograms demonstrating distributions of composite clinical outcome scores at 8 and 24 weeks](image)

**Table 10.6: Correlations between outcome scores and percentage change in other measures**

<table>
<thead>
<tr>
<th>Variable 1</th>
<th>Variable 2</th>
<th>Spearman's Rho</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>8 week composite clinical outcome score</td>
<td>SGRQ</td>
<td>-0.21</td>
<td>p=0.04</td>
</tr>
<tr>
<td></td>
<td>6MWT</td>
<td>0.26</td>
<td>p=0.005</td>
</tr>
<tr>
<td></td>
<td>Hb</td>
<td>0.25</td>
<td>p=0.006</td>
</tr>
<tr>
<td>24 week composite clinical outcome score</td>
<td>SGRQ</td>
<td>-0.26</td>
<td>p=0.03</td>
</tr>
<tr>
<td></td>
<td>6MWT</td>
<td>0.27</td>
<td>p=0.02</td>
</tr>
<tr>
<td></td>
<td>Hb</td>
<td>0.46</td>
<td>p=0.04</td>
</tr>
</tbody>
</table>

**DICHOTOMOUS SCORE**

Dichotomous scores were available in 127 study participants at 8 weeks and 88 study participants at 24 weeks (87 matched pairs); a positive score, indicating success in achieving at least 10% increase in body weight plus at least 10% increase in lung
function or improvement in respiratory symptoms, was achieved by only 14.2% of participants by 8 weeks, and 43.2% by 24 weeks (Table 10.6).

Positive scores at 8 and 24 weeks were significantly associated with greater improvements in SGRQ score; also at 8 weeks, in greater improvement in 6-minute walk distance, and at 24 weeks, in rise in haemoglobin (all p values<0.05, Rank-Sum tests). Other associations were not significant. The dichotomous score at 8 weeks was not associated with 8 week sputum AFB status. The dichotomous score at 24 weeks could not be compared with outcome since those with unfavourable outcomes (i.e. died, defaulted or were transferred), did not reach week 24. The individual who failed treatment did not achieve a positive score by week 24.

**ADVERSE EVENT REPORTING**

Five serious adverse events (SAE) occurred: 2 deaths and 3 hospitalisations (Table 10.8). In each case a detailed clinical summary was provided to the Data and Safety Monitoring committee. All SAE were judged to be unlikely to be related, or unrelated, to the study medications (L-arginine / vitamin D / placebo L-arginine / placebo vitamin D) according to GCP definitions (provided on the SAE reporting guide, see Appendix 15.9).

Non-serious adverse events (any new symptom not reported at baseline) were very common, being reported in 128 of the 139 study participants (92.1%) who had been enrolled for at least 8 weeks. The most common AEs (Figure 10.8) were itch (reported at least once in 47.5% of people) followed by gastrointestinal disturbance (nausea, vomiting, abdominal pain, bloating, diarrhoea, epigastric pain and/or constipation), reported by 46.8% of all study participants, peaking at week 1 and 2 after commencing treatment. Of the gastrointestinal symptoms, nausea was most common, followed by bloating then vomiting.

While common, adverse events were mostly mild, often requiring reassurance only or symptomatic management (analgesics, antacid medication etc). However among the 139 participants who had 8-week follow up data, one case of severe desquamating rash occurred, with recurrence on re-challenge with both ethambutol then pyrazindamide,
necessitating a change of regimen. Hence the rate of major anti-tuberculosis drug reactions requiring regimen switch was 1/139 (0.7%).

Notably, 12 study participants developed symptomatic malaria (*7 Plasmodium falciparum, 5 Plasmodium vivax*) whilst undergoing TB treatment. Malaria diagnosis (blood film) and treatment (dihydroartemisinin piperaquine ± primaquine) was provided in keeping with Timika protocols.

HIV positive status was associated with a significantly higher likelihood of vomiting (HIV+: 27.8%, HIV-: 9.2%, p=0.04) and bloating (HIV+: 33.3%, HIV-: 14.2%, p=0.05), and somewhat higher likelihood of malaise (HIV+: 27.8%, HIV-: 11.7%, p=0.08) during the first 8 weeks of treatment. Other adverse events, and overall likelihood of AE, were not significantly higher in HIV-TB co-infected participants.

**Figure 10.8: Non-serious adverse events experienced by AVDAPT study participants**

![Graph showing percentage of participants experiencing various symptoms](image-url)
Table 10.7: Serious adverse events (SAE)

<table>
<thead>
<tr>
<th>Age (yrs)</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male</td>
<td>Male</td>
<td>Female</td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td>Ethnicity</td>
<td>Papuan</td>
<td>Papuan</td>
<td>Non-Papuan</td>
<td>Non-Papuan</td>
<td>Non-Papuan</td>
</tr>
<tr>
<td>HIV status</td>
<td>Positive</td>
<td>Positive</td>
<td>Positive</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>SAE type</td>
<td>Death at home</td>
<td>Death in hospital</td>
<td>Hospitalisation</td>
<td>Hospitalisation</td>
<td>Hospitalisation</td>
</tr>
<tr>
<td>Diagnosis</td>
<td>Progressive respiratory illness due to TB or secondary pneumonia</td>
<td>Stroke complicated by aspiration pneumonia</td>
<td>Pneumothorax</td>
<td>Vomiting and dehydration</td>
<td>MDR-TB with progressive pulmonary disease and large pleural effusion</td>
</tr>
<tr>
<td>Time of SAE (weeks after TB treatment start)</td>
<td>7</td>
<td>9</td>
<td>4</td>
<td>3</td>
<td>16</td>
</tr>
<tr>
<td>Relatedness of SAE to study drugs</td>
<td>Unlikely</td>
<td>Unlikely</td>
<td>Unlikely</td>
<td>Unlikely</td>
<td>Unrelated</td>
</tr>
<tr>
<td>SAE resulted in study medication cessation?</td>
<td>No</td>
<td>Already ceased</td>
<td>No</td>
<td>Yes</td>
<td>Already ceased</td>
</tr>
<tr>
<td>SAE Outcome</td>
<td>Died</td>
<td>Died</td>
<td>Recovery; cured</td>
<td>Recovery; cured</td>
<td>Recovery; 2nd-line treatment ongoing</td>
</tr>
</tbody>
</table>

Hypercalcaemia, defined as a non-serious adverse event (unless resulting in hospitalisation, life-threatening illness or death), occurred in 23 / 139 (16.5%) people (Table 10.9), most commonly in week 2 (42.9% in week 2, 35.7 in week 4, 21.4% in week 8). None were symptomatic or required specific treatment or hospitalisation.

While 1 person had mild to moderate asymptomatic hypercalcaemia sustained over 5 weeks (Figure 10.9), and 2 people had elevated calcium readings on 2 occasions each,
the remaining 20 people with hypercalcaemia had only a single reading greater than the normal range.

Table 10.8: Number of study participants with hypercalcaemia

<table>
<thead>
<tr>
<th>Hypercalcaemia: no. (%)</th>
<th>Study participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>139</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hypercalcaemia</th>
<th>Study participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (ionised Ca &lt;1.33 mmol/L)</td>
<td>116 (83.5)</td>
</tr>
<tr>
<td>Mild (1.33-1.39 mmol/L)</td>
<td>18 (13.0)</td>
</tr>
<tr>
<td>Moderate (1.40-1.49 mmol/L)</td>
<td>5 (3.6)</td>
</tr>
<tr>
<td>Severe (&gt;1.50 mmol/L)</td>
<td>0 (0.0)</td>
</tr>
</tbody>
</table>

Figure 10.9: Serial calcium readings in a representative individual receiving TB treatment and study medications

Overall, calcium increased by a small but statistically significant amount (but within the normal range i.e. <1.33 mmol/L) from 1.21 to 1.24 mmol/L between weeks 0 to 2, then remained elevated during the remainder of the intensive phase (Figure 10.10).

Figure 10.10: ionised calcium (mean indicated by bar and range) by week in AVDAPT study participants

*p value calculated using paired t-test. The differences between weeks 2 and 4 or 8 were not significant.
INTERIM ANALYSIS

AE potentially related to study drugs were assessed in the interim safety analysis performed by the statistician of the Data and Safety Monitoring Committee. Data from 111 study participants (25% of planned sample) were evaluated. As shown in Table 10.9, study groups were balanced in number and in most characteristics except for gender. No differences in rates of adverse events (hypercalcaemia, or new symptoms of gastrointestinal, neurological or respiratory origin) were observed between the 4 study arms.

Table 10.9: Summary of findings of the interim analysis

<table>
<thead>
<tr>
<th></th>
<th>Group A</th>
<th>Group B</th>
<th>Group C</th>
<th>Group D</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline characteristics</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n</td>
<td>28</td>
<td>29</td>
<td>27</td>
<td>27</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Age in yrs: mean</td>
<td>31.8</td>
<td>31.4</td>
<td>30.4</td>
<td>29.0</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Female: no. (%)</td>
<td>7 (25.0)</td>
<td>14 (48.3)</td>
<td>13 (48.2)</td>
<td>5 (18.5)</td>
<td>p=0.03</td>
</tr>
<tr>
<td>Papuan: %</td>
<td>50.0</td>
<td>44.8</td>
<td>48.2</td>
<td>48.2</td>
<td>p=0.98</td>
</tr>
<tr>
<td>Weight in kg: mean</td>
<td>47.8</td>
<td>48.3</td>
<td>47.2</td>
<td>48.4</td>
<td>p=0.98</td>
</tr>
<tr>
<td>HIV+: no.</td>
<td>3</td>
<td>8</td>
<td>2</td>
<td>1</td>
<td>p=0.08</td>
</tr>
<tr>
<td>MDR-TB</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>p=0.9</td>
</tr>
<tr>
<td>Haemoglobin in g/dL: mean</td>
<td>12.0</td>
<td>11.6</td>
<td>12.3</td>
<td>12.7</td>
<td>p=0.4</td>
</tr>
<tr>
<td><strong>Adverse events</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Serious adverse event: no.</td>
<td>1</td>
<td>1</td>
<td>2</td>
<td>0</td>
<td>p=0.3</td>
</tr>
<tr>
<td>Hypercalcaemia: %</td>
<td>12.0</td>
<td>11.6</td>
<td>12.3</td>
<td>12.7</td>
<td>p=0.4</td>
</tr>
<tr>
<td>Any new gastrointestinal symptoms: %</td>
<td>39.3</td>
<td>44.8</td>
<td>37.0</td>
<td>48.2</td>
<td>p=0.8</td>
</tr>
<tr>
<td>Any new neurological symptoms: %</td>
<td>32.1</td>
<td>17.2</td>
<td>14.8</td>
<td>7.4</td>
<td>p=0.1</td>
</tr>
<tr>
<td>Any new respiratory symptoms: %</td>
<td>28.6</td>
<td>31.0</td>
<td>29.6</td>
<td>37.0</td>
<td>P=0.9</td>
</tr>
</tbody>
</table>

*p values were calculated using Fisher’s exact or Pearson’s Chi2 tests.

Members of the Data and Safety Monitoring committee concluded that results of the interim safety analysis revealed no concerns, and deemed the study safe to proceed.

10.4 DISCUSSION

The chief findings from analyses of longitudinal data in this chapter are that losses to follow up were modest, treatment outcomes were close to WHO targets, predictors of
bacteriological outcomes were consistent with previous reports in the literature, and no safety concerns for participants randomised to adjunctive therapy were identified. Furthermore, the 12-point clinical score developed as a primary outcome measure of the study is shown to be an appropriate measure of clinical improvement.

**SAMPLE SIZE AND LOSSES TO FOLLOW UP**

Assumptions which guided the AVADPT sample size calculation (see Methods, Chapter 6) included a 20% reduction in the proportion culture positive at one month (from 60% to 40%) with losses to follow up of 10%. In the current data, the 1-month culture positive rate of 42.1% includes participants in all 4 study arms (3 intervention arms, one placebo arm), and is therefore within the anticipated range on which the sample size calculation was based. Losses to follow up by 1 month were less than anticipated (5.8%). People who were lost to follow up were representative of the group as a whole, but with a non-significant trend towards more extensive radiological disease extent and worse lung function at baseline.

**BACTERIOLOGICAL RESULTS**

The estimation that 60% of Timika patients might remain culture positive at 1 month was made on the basis of previous data from Timika indicating high bacillary burden and cavitary disease, and from other studies showing mean time to culture negativity (in mostly drug sensitive TB) of 32 to 57 days. Other published comparative figures include 11.1% of smear-positive pulmonary TB patients remaining culture positive at 2 months in one study, and 38.6% at 1 month and 15.7% at 2 months in another study. Our reported rates of culture positivity at 1 month (42.1%) and 2 months (19.6%) are somewhat higher, possibly related to relative disease severity at baseline among the patients in these different settings.

However in examining predictors of sputum smear and culture conversion in these study participants, it is apparent that while expected variables such as baseline AFB grade and presence of cavities were predictive of smear conversion and culture at 4 weeks, factors predicting 8-week culture conversion were less readily evident. AFB smear grade at baseline did remain predictive of 8-week culture conversion, but the effect was relatively small (odds ratio 1.9). This result could suggest that other
determinants, such as the robustness of an individual’s anti-mycobacterial immune response, potentially influenced by factors such as serum vitamin D or L-arginine levels, might have relevance in determining culture conversion. This will be evaluable later in the course of the AVADPT study.

Presence of cavitary disease and higher AFB density at TB diagnosis are well recognised to predict slower sputum smear conversion times.\textsuperscript{142, 150, 151, 213} Additional predictors of sputum smear conversion rates in one study included older age, male sex and smoking,\textsuperscript{151} but these associations have not been confirmed elsewhere,\textsuperscript{142, 150, 213, 214} nor in the data presented in this chapter. Also consistent with the findings presented here, isoniazid resistance has not been shown to influence sputum smear conversion rates\textsuperscript{142} or other outcome measures\textsuperscript{215} among people receiving standard isoniazid-containing regimens. HIV has been found to be associated with either no difference\textsuperscript{150} or with shorter times to smear conversion,\textsuperscript{213} again supporting the current findings in which a small but non-significant faster smear conversion during the early treatment phase was noted among HIV positive people, likely to be attributable to their lower baseline smear grade and lower likelihood of cavitary disease.

Time to smear conversion is an important public health measure due to the reliance on this measure, in many protocols, to guide duration of respiratory isolation, and the duration of intensive phase treatment. In Timika, according to standard practice, intensive phase treatment (the daily 4-drug regimen) is continued for an extra month if the sputum smear is still positive at week 8. However in this study as elsewhere, persistent smear positivity can over-estimate infectivity risk, due to persistence of non-viable or minimally viable bacilli in sputum.\textsuperscript{212} Here, around half (54\%) of smear positive 8-week sputum specimens were culture negative. The most likely explanation is that the visualised bacilli were non-viable, but other explanations include loss of viability prior to the specimen reaching the laboratory, excessive de-contamination procedures or other potential laboratory errors (e.g. labelling or data entry error). These results confirm that the use of smear status to guide the duration of respiratory precautions may be an excessively cautious approach; however, in the Timika setting, delays in culture and sensitivity turn-around time, and the potential for false-negative culture results, mean that this approach remains sensible.
TREATMENT OUTCOMES

Key targets set by the WHO for DOTS programs are to detect at least 70% of new sputum smear-positive TB cases, and cure at least 85% of these, where cure is defined as negative smears in the last month of treatment plus on at least one previous occasion. People who complete treatment but cannot be proven to be cured, because of absence of a second confirmatory negative sputum sample, are nevertheless classified as achieving a successful outcome. WHO TB annual reports consider the 85% target to be fulfilled if 85% attain treatment success (cure or completion, rather than cure alone).

Cure was achieved in 78.2% of AVDAPT study participants, and overall treatment success in 84.5%. These data, only just reaching WHO targets, indicate the difficulties of confirming cure and retaining patients in care, even in a well-functioning DOTS setting. (The Timika TB clinic is considered to achieve better outcomes than other Puskesmas clinics and private practitioners in Timika.) Furthermore, study participants comprise only those well enough to be managed as outpatients at diagnosis, with the most unwell TB patients requiring initial hospital care excluded from this RCT. However, as the large majority of people with TB in Indonesian and globally are managed on an outpatient basis (an important component of nosocomial transmission reduction strategies), the disease severity of these study participants is therefore representative of the majority of Indonesian pulmonary TB cases.

Comparing with the most recently available national Indonesian reported outcomes (cure 83%, completed 8.5%, died 2.1%, failed 0.6%, defaulted 4.6%, transferred 1.7%), transfer and default rates were higher in this study, likely to be attributable to the high mobility of the Timika population. Death rates were low compared with nations with higher HIV-TB co-infection or MDR-TB rates (e.g. South Africa: 7.3%, Russian federation 12%). Direct comparison with national statistics may be somewhat misleading, since the careful scrutiny of each case in this study to determine that outcome is correctly classified may result in different reported outcomes compared with national reporting seeking to satisfy WHO targets. Indeed, in Indonesia overall in 2006, treatment success was reported to have been achieved in a surprisingly high 91% of cases.

The WHO treatment outcome categories serve as an important guide in low-income countries where routine culture facilities are unavailable, providing an indication of DOTS program performance. They are less useful as a clinical measure at the level of
the individual, as has been previously acknowledged;\textsuperscript{216} for instance, cause of death, which may be unrelated to TB, is not taken into account. Therefore it is not surprising that in the data examined here, no firm associations were identified between favourable / unfavourable outcome and other markers of disease severity, apart from illness duration prior to diagnosis being longer in the 2 people who died and the one who failed treatment.

An additional important outcome measure for trials of TB treatment is TB relapse in the 24 months following the completion of treatment.\textsuperscript{217} Relapse is not assessed in the AVDAPT study because the outcomes of interest are impacts of adjunctive therapies on early treatment responses, because 2-month sputum culture results correlate strongly with later relapse risk,\textsuperscript{145, 217} and because the additional follow up time and MTB isolate typing which would be required to distinguish relapse from reinfection, pose feasibility problems at the research site.

**DEVELOPMENT OF A COMPOSITE CLINICAL OUTCOME SCORE**

Use of a composite clinical outcome score as a primary outcome measure of the AVDAPT study was considered to be prudent in order to avoid reliance on a single primary outcome measure requiring off-site processing in a reference laboratory, and to capture important clinical outcomes independent of bacteriological results. The resulting 12-point score, comprising components which were selected \textit{pre hoc} on the basis of perceived clinical relevance and established importance in TB, was found to be significantly related to changes in other treatment response measures, and to decrease significantly over time. Furthermore, scores were broadly distributed at the two time points (8 and 24 weeks) indicating that this score is likely to provide adequate discriminatory capacity.

The dichotomous score shows evidence of being too stringent and therefore is less likely to be of good discriminatory value in assessing AVDAPT study outcomes. Positive (good) scores on the dichotomous measure were only achieved in 14\% of patients by 8 weeks, and less than half by treatment completion, including in the majority of people who had a successful treatment outcome. Therefore this analysis establishes that the ordinal score is likely to be of more value than the dichotomous
score, and hence the former will be employed as the AVDAPT study’s second primary endpoint.

**Adverse events**

Transient, asymptomatic hypercalcaemia was documented in 16.5% of study participants between 2 and 8 weeks, not more than has been reported elsewhere during the course of TB treatment with or without vitamin D supplementation.\(^\text{87, 158}\) In other recent published reports of vitamin D supplementation in TB, 11 TB patients were administered a single dose of 100 000 IU ergocalciferol, resulting in no episodes of hypercalcaemia at 8 weeks,\(^\text{86}\) and in a randomised trial of 100 000 IU cholecalciferol in patients with TB in Guinea-Bissau, there was no difference in hypercalcaemia symptoms or detection of biochemical hypercalcaemia in those randomised to the treatment arm.\(^\text{81}\) Other studies have also reported safe administration of vitamin D in TB.\(^\text{157, 159}\)

Although numbers in each study arm were small at the time of the interim analysis, our interim safety analysis also indicated that hypercalcaemia and other adverse event rates did not differ between the study arms. Therefore this new data adds to the recently-growing body of literature indicating the safety of vitamin D use in active TB, in contrast to the view still mentioned in some contemporary guidelines that active TB contraindicates vitamin D administration.\(^\text{218}\) High rates of hypercalcaemia reported in 1984 by Narang et al\(^\text{219}\) in vitamin D-supplemented people with or without TB formed the basis of these concerns, but these findings have not been reproduced, and are speculated to have arisen due to a major underestimation of the actual amount of vitamin D administered in this study.\(^\text{220}\) A second reason to cite TB as a contraindication to vitamin D treatment might be the hypothetical concern of vitamin D-induced up-regulation of host immune responses leading to an immune reconstitution-type effect with transient worsening of TB symptoms. Such concerns have not been borne out by studies to date,\(^\text{74, 81, 86, 157, 159}\) nor in the data presented herein, (specifically, worsening of respiratory symptoms was not significantly higher in any of the treatment arms). The cell-mediated immune depressive effect of vitamin D\(_3\) (in contrast to its stimulatory effect on innate immune responses) might in fact be hypothesised to act to prevent excessive cell-mediated immune responses and thereby mitigate inflammatory immunopathology.\(^\text{38}\)
Adverse effects of oral L-arginine at the dose used in this study (6g daily) include diarrhoea, bloating or nausea, but these are uncommon, affecting only about 3 - 11% of people. Gastrointestinal symptoms affected almost half (46.8%) of the study participants overall, but were usually mild. Rates were approximately even in each study arm (between 37.0 and 48.2% at the time of the interim analysis), indicating no current evidence for additional gastrointestinal upset associated with L-arginine. True differences might have been undetectable due to the small numbers in each arm (27 to 29 people) and will be re-evaluated when 50% and 100% of participant data is available. Similarly, the uneven gender distribution due to the small numbers of females in the study (only 5 to 14 in each arm) may balance as the sample size increases. Randomisation is stratified on the basis of ethnicity but not gender, and the randomisation process has been successful in balancing all other baseline characteristics and maintaining even numbers among study arms.

Gastrointestinal disturbance, as well as being a potential study medication adverse effect, is also widely reported with anti-tuberculosis antibiotics, either as a direct medication side effect, or secondary to drug-induced hepatitis. However gastrointestinal disturbance rates are usually quoted as occurring in only around 1-10% of people taking anti-tuberculosis medications. The higher rates reported here may be attributable to the active surveillance undertaken, with weekly questioning about symptoms, which may encourage people to report mild symptoms they might usually not disclose. The rate of drug regimen switching due to side effects was not higher than expected (0.7%, compared with 5.1% reported elsewhere), confirming the mild nature of most of the AE reported here.

10.5 CONCLUSION

Confirmation of previously-defined predictors of sputum smear and culture conversion provides confidence in the reliability of the methods used in the AVADPT study to grade sputum microscopy, perform MTB culture and classify the presence of cavitary disease on x-ray, as well as in the record-keeping and data entry processes employed. The observation that predictors of culture conversion become less important over the course of 8 weeks suggests the potential importance of other as-yet undefined factors in determining an individual’s response to therapy. After evaluating two alternative
methods for coding the second primary endpoint, a composite clinical endpoint which allocates a total score between 0 and 12 to study participants, depending on their percentage improvement in weight and respiratory parameters, has been selected for use in the trial. The interim analysis and the Data and Safety Monitoring committee’s recommendation adds weight to the argument that vitamin D at the dose used is safe in TB. Additionally, the administration of oral L-arginine in active TB appears to be associated with no apparent safety concerns.
11 Results V: Exhaled nitric oxide in pulmonary TB

In this chapter I describe the performance of the portable NiOX MINO® analyser for measurement of fractional exhaled nitric oxide (FeNO), and examine FeNO characteristics in AVDAPT study participants and healthy volunteers in Timika.

11.1 INTRODUCTION

FeNO in Pulmonary TB

As detailed in Chapter 4, nitric oxide (NO) is a key component of the macrophage antimycobacterial immune response, and is measurable in expired air using chemiluminescence analysers. Immunological, biochemical and haemodynamic determinants of NO in expired air are multiple, complex and incompletely defined, but primarily include macrophage activation, Nitric oxide synthase 2 (NOS2) production, and availability of NOS2 substrate (L-arginine).

Core hypotheses of the AVDAPT study include that baseline pulmonary NO production will be elevated in pulmonary TB but inversely associated with disease severity, that baseline and post-treatment increments in exhaled NO will be associated with rapidity and magnitude of the treatment response, and that L-arginine supplementation in pulmonary TB will enhance pulmonary production of NO. In this chapter I therefore compare FeNO in TB patients versus healthy controls, investigate the relationships between baseline FeNO and TB disease severity measures, and describe longitudinal trends in FeNO. The impact of L-arginine supplementation on FeNO will be evaluated at trial completion when the randomisation code has been revealed.

Performance characteristics of NiOX MINO FeNO analyser

A NiOX FLEX® non-portable exhaled NO analyser, considered the reference analyser, is installed at the Timika Research facility, housed in a custom-built, temperature-regulated room at 23°C. A handheld NiOX MINO® exhaled NO analyser is available.
for use by participants in the AVDAPT study, who are evaluated at the Timika TB clinic, 15 km from the Timika Research facility. NiOX MINO and FLEX analysers have been shown to provide comparable readings under controlled research settings (see Chapter 3).\textsuperscript{101-103} The portable analyser has a replaceable sensor providing a set number of tests (100 or 300 tests depending on the sensor type). It does not permit calibration, and therefore requires regular comparison against the reference analyser.

The manufacturer’s guidebook states that NiOX MINO precision (repeatability) is ±5 ppb of measured values <50 ppb, or ±10\% of measured values ≥50 ppb, and accuracy (compared with gold standard) should be <3 ppb of measured values <30 ppb or <10\% of measured values ≥30 ppb.\textsuperscript{222} The optimal environmental conditions for NiOX MINO® sensor operation include an ambient temperature range of 16 to 30°C, and relative humidity of 20 to 60\%.\textsuperscript{222} Performance characteristics of the portable analyser sensors have not previously been described in tropical settings.

Potential sources of inaccuracy in \(\text{FeNO}\) measurement in AVDAPT study participants require consideration and, if necessary, correction. These include any systematic difference in results between the portable and reference analysers, or drift within NiOX MINO sensors over time, which has been noted by other investigators.\textsuperscript{223} Therefore prior to presenting \(\text{FeNO}\) data, the results of quality control measurements to determine the need for correction factors are presented.

\textbf{11.2 METHODS}

Methods of study participant recruitment are as described in Chapter 6. The NiOX FLEX and handheld NiOX MINO exhaled NO analysers are operated according to the guidelines provided by the manufacturer (Aerocine) and the American Thoracic Society.\textsuperscript{100} The NiOX FLEX machine is calibrated fortnightly using nitric oxide (200 ppB), and is serviced regularly by the manufacturer. All \(\text{FeNO}\) measurements from study participants or healthy controls are made using the NiOX MINO, since the TB clinic where they are seen is in a different location from the research building housing the NiOX FLEX.

\(\text{FeNO}\) is measured once on the NiOX MINO analyser in healthy controls, and in TB patients, at weeks 0, 1, 2, 4, 8 and 24. Subjects are asked to refrain for eating and
smoking for several hours prior to FeNO measurement, and timing of the last meal is recorded. To measure FeNO, subjects inhale NO-free air to total lung capacity by inhaling through a mouthpiece containing an NO scrubber, then exhale for a 10-second duration at a constant flow rate of 50 ± 5 mL/s, achieved by real-time audio and visual feedback from the measuring device indicating whether the flow rate is correct.

An additional historical dataset of FeNO values in healthy controls was also made available for this analysis. This dataset comprises 44 Papuan and 4 Non-Papuan volunteers recruited in 2005 at the Timika research facility as the control group for a study of FeNO in subjects with malaria, by NIHRD-MSHR research staff led by Dr Tsin Yeo. FeNO was measured on a single occasion using the NiOX FLEX (reference) analyser.

**QUALITY CONTROL (QC) TESTING AND ANALYSIS**

FeNO values obtained using the portable analyser (MINO) used in the TB clinic are compared with the reference analyser (FLEX) at the research building once weekly. QC measures comprise the following 3 methods:

1. FeNO readings are obtained sequentially from healthy human biological controls (AVDAPT study staff members) on both machines at the research building, with measures made not more than 15 minutes apart. Eating and exercising are avoided for 2 hours prior to FeNO measurement.

2. FeNO readings are obtained using an impermeable foil bag filled with a mixture of exhaled breath and NO from a standard 200ppb NO cylinder, passed into the reference analyser then the portable analyser. This method permits higher readings than can be obtained from the available biological controls (but within the range obtained among study participants). Foil bags and accompanying tubing and 3-way taps were provided by Dr Cheryl Salome, Woolcock Institute of Medical Research, NSW.

3. In the instance of the reference analyser being unavailable (faulty or under service), ongoing weekly measures from the healthy human biological controls, whose usual FeNO readings are known, are continued on the NiOX MINO.

Statistical analyses were performed in Stata 10.1, with graphs produced either in Stata or Graph Pad Prism 5.0. Regression analyses were used to calculate correction factors
and examine associations between $\text{FE}_{\text{NO}}$ and predictor variables. Bland-Altman plots were constructed to evaluate differences in $\text{FE}_{\text{NO}}$ results within and between analysers. Proportions were compared between groups using Pearson’s $\chi^2$ tests. Differences in continuous data between groups were tested using 2-sample $t$-tests for normally distributed data, and the Wilcoxon Rank-Sum test for non-normally distributed data. Paired Students $T$-tests were used to compare $\text{FE}_{\text{NO}}$ results obtained at 2 time points from the same individual (log-transformed since $\text{FE}_{\text{NO}}$ data was non-parametrically distributed). Statistical tests were two-sided, with significance for $p$-value $<0.05$. Correlations between non-parametric data were examined using Spearman’s method.

11.3 RESULTS

**QUALITY CONTROL DATA**

$\text{NiOX MINO}$ precision (repeatability)

Precision of $\text{FE}_{\text{NO}}$ measures was ascertained from repeated measures made using a single analyser and paired measures using 2 different NiOX MINO analysers. The average difference between repeated measures of exhaled nitric oxide made within 15 minutes by an individual on a single NiOX MINO analyzer was $-0.4$ ppb (95% limits of agreement $-6.2$ to $5.5$) calculated using the Bland-Altman method (Table 11.1 and Figure 11.1). The average difference between paired measures made within 15 minutes by an individual on two different NiOX MINO analyzers (with different sensors) was $0.8$ ppb (95% limits of agreement $-4.3$ to $5.8$) (Table 11.1 and Figure 11.2).

<table>
<thead>
<tr>
<th></th>
<th>$n$</th>
<th>Bland-Altman method</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Difference between readings in ppb: mean (95% limits of agreement)</td>
<td>Pearson’s R</td>
</tr>
<tr>
<td>Repeated measures on one analyser</td>
<td>36</td>
<td>$-0.4$ (-6.2 to 5.5)</td>
<td>0.81</td>
</tr>
<tr>
<td>Paired measures on two analysers</td>
<td>16</td>
<td>$0.8$ (-4.3 to 5.8)</td>
<td>0.82</td>
</tr>
</tbody>
</table>

Table 11.1: Correlation between $\text{FE}_{\text{NO}}$ measures
Table shows correlations between sequential FENO measures from biological controls (research staff members) on one NiOX MINO analyser, and paired measures on two analysers.
**NiOX MINO accuracy (comparison with ‘gold standard’)**

$F_{ENO}$ values obtained from the Niox Mino portable analysers correlated highly with values obtained from the Niox Flex ‘gold standard’ analyser ($R=0.95$, $p<0.0001$), but were consistently lower, with the difference being proportional to $F_{ENO}$ values (see Bland Altman plot, Figure 11.3), indicating the need for a correction factor. For example, as demonstrated in the scatter plot (Figure 11.4), for true $F_{ENO}$ values of 20 and 80 ppb, the NiOX MINO gave readings of approximately 15 and 50 ppb respectively.
Examination of the difference in $F_{ENO\text{MINO}}$ and $F_{ENO\text{FLEX}}$ values demonstrated that readings were almost identical between the two analysers for the first month of the study (June 12 2008 – July 7 2008), but lower readings were consistently obtained from all MINO sensors thereafter (July 8 2008 – October 30 2009), postulated to be due to local environmental conditions (high temperature and humidity). All efforts to maintain optimal operating environments were instigated at this point, including storing the portable analyser and its sensors in a dry, air-conditioned environment at all times that the analyser was not in use, and placing it in a 19°C refrigerator between tests at the TB clinic.

Additionally in the case of 2 NiOX MINO sensors, a significant downward drift was noted over time, generating spuriously low readings, including frequent readings less
than the lower limit of detection (5 ppb), and culminating in sensor failure in both instances before all tests on the sensor had been used. Therefore $\text{FE}_\text{NO}$ data for TB patients and healthy controls collected during these two time periods (14/7/08 - 7/9/08 and 17/03/09 - 24/03/09) prior to sensor failure were culled from further analyses.

**Correction factor applied to NiOX MINO results**

Correction factors to apply to $\text{FE}_{\text{NOMINO}}$ values obtained at the TB clinic, to allow an approximation of ‘true’ ($\text{FE}_{\text{NOFLEX}}$) values, were calculated using the equation for a straight line:

$$y = a + bx$$

where

- $y = \text{FE}_{\text{NOMINO}}$ (test value)
- $x = \text{FE}_{\text{NOFLEX}}$ (true value)
- $a = \text{constant derived from regression of MINO sensor on FLEX}$
- $b = \text{coefficient derived from regression of MINO sensor on FLEX}$

Solving for $x$:

$$\text{True } \text{FE}_{\text{NO}} = \frac{\text{FE}_{\text{NOMINO}} - a}{b}$$

To avoid extrapolating far beyond the bounds of the quality control range of $\text{FE}_{\text{NO}}$ data (Figure 11.4) or beyond the bounds of physiological likelihood, the maximum $\text{FE}_{\text{NOMINO}}$ value was considered to be 100 ppb, to which corrections were subsequently applied. This affected the results of 2 healthy controls but no TB patients. Corrections are summarised in Table 11.2.

<table>
<thead>
<tr>
<th>Time period</th>
<th>Correction applied to NiOX MINO $\text{FE}_{\text{NO}}$ result</th>
</tr>
</thead>
<tbody>
<tr>
<td>12/06/08 – 07/07/08</td>
<td>$\text{True } \text{FE}<em>{\text{NO}} = \frac{(\text{FE}</em>{\text{NOMINO}} - 0.2795245)}{0.9560819}$</td>
</tr>
<tr>
<td>08/07/08 – 30/10/09</td>
<td>$\text{True } \text{FE}<em>{\text{NO}} = \frac{(\text{FE}</em>{\text{NOMINO}} - 2.922176)}{0.5824131}$</td>
</tr>
<tr>
<td>Data not analysed due to faulty sensor</td>
<td>Data coded as missing</td>
</tr>
<tr>
<td>17/03/09 - 24/03/09</td>
<td>Data coded as missing</td>
</tr>
<tr>
<td>$\text{FE}_{\text{NOMINO}} &gt;100$</td>
<td>$\text{FE}_{\text{NOMINO}} =100$</td>
</tr>
</tbody>
</table>
**FE\textsubscript{NO} in adults with pulmonary TB versus healthy subjects**

Table 11.3: Characteristics and FE\textsubscript{NO} in healthy volunteers and TB patients at enrolment

<table>
<thead>
<tr>
<th></th>
<th>Healthy volunteers</th>
<th>TB patients</th>
<th>P value*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>N</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dataset 1</td>
<td>83</td>
<td>162</td>
<td>-</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>48</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>35</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Age in yrs: median (range)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dataset 1</td>
<td>27 (18-65)</td>
<td>27 (15 – 65)</td>
<td>p=0.4</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>27.5 (18 – 42)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>27 (18-65)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Female: no. (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dataset 1</td>
<td>26 (31.3)</td>
<td>54 (33.3)</td>
<td>p=0.5</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>17 (35.4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>9 (25.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Papuan: no. (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dataset 1</td>
<td>62 (74.4)</td>
<td>78 (48.2)</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>44 (91.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>18 (51.4)†</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Current or ex-smoker: no. (%)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dataset 1</td>
<td>42 (51.9)</td>
<td>93 (57.4)</td>
<td>p=0.3</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>26 (56.5)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>16 (45.7)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Weight in kg: mean (SD)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dataset 1</td>
<td>60.8 (9.5)</td>
<td>48.4 (7.5)</td>
<td>p&lt;0.0001</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>59.8 (9.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>62.2 (10.2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Height in m: mean (SD)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dataset 1</td>
<td>1.61 (0.07)</td>
<td>1.58 (0.08)</td>
<td>p=0.1</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>-</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.61 (0.07)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Number with corrected FE\textsubscript{NO} available</strong></td>
<td>83</td>
<td>133</td>
<td></td>
</tr>
<tr>
<td><strong>FE\textsubscript{NO} in ppb‡: median (range)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dataset 1</td>
<td>16.9 (2.5-166.7)</td>
<td>15.6 (2.5 – 106.6)</td>
<td>p=0.4</td>
</tr>
<tr>
<td>Dataset 2</td>
<td>16.6 (4.9 – 64.6)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>19.0 (2.5-166.7)§</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*P values calculated from Pearson’s chi2 tests, 2-sample t-tests or rank-sum tests. †Proportion of Papuans in healthy volunteer Dataset1 vs Dataset2: p<0.0001. ‡Corrected FE\textsubscript{NO} used the calculated correction factors applied to Dataset 2 healthy volunteers and TB patients. §FE\textsubscript{NO} in healthy volunteer Dataset1 vs Dataset2: p=0.6

**Healthy subjects**

FE\textsubscript{NO} results were available for 48 healthy volunteers recruited in 2005 (Dataset 1) who had FE\textsubscript{NO} measured using the NiOX FLEX. FE\textsubscript{NO} results were also available for 35 healthy volunteers recruited in 2008-9 (Dataset 2) who had FE\textsubscript{NO} measured using the NiOX MINO. The proportion of Papuans to Non-Papuans was significantly higher in
Dataset 1 than Dataset 2, and median $\text{FeNO}$ trended higher in Dataset 2 (19.0 vs 16.6), but the difference was not significant (Table 11.3). Height was not recorded in Dataset 1.

**Predictors of $\text{FeNO}$ in healthy volunteers**

No variables were found to significantly predict $\text{FeNO}$ among the pooled or individual healthy volunteer groups. Statistically non-significant differences included that smokers had lower median $\text{FeNO}$ (15.5 ppb) than non-smokers (17.3 ppb, $p=0.08$), and people of Papuan ethnicity had higher median $\text{FeNO}$ (17.3 ppb) than Non-Papuans (12.2 ppb, $p=0.08$); these associations diminished further in significance when controlling for each other and age, sex, weight and height. Notably, weight and height failed to significantly predict $\text{FeNO}$ (regression coefficient for weight: -0.004, $p=0.7$; for height: -0.18, $p=1.0$). $\text{FeNO}$ was also found to be independent of age, sex and haemoglobin.

**$\text{FeNO}$ in TB patients versus healthy volunteers**

At the time of TB diagnosis, no statistically significant difference was identifiable between people with TB and healthy volunteers. People with TB had somewhat lower median $\text{FeNO}$ than healthy volunteers (see Table 11.3), particularly when comparing TB patients with the new healthy volunteer sub-set (Dataset 2, 2008-9) whose $\text{FeNO}$ measurements were made using the same NiOX MINO method and application of correction factors as the AVDAPT study participants. Although these $\text{FeNO}$ difference was not statistically significant at week 0 ($p=0.2$, 2-sample t-test on log-transformed $\text{FeNO}$), $\text{FeNO}$ decreased further in TB patients immediately after starting treatment, such that by weeks 1 and 2 of TB treatment, patients had significantly lower $\text{FeNO}$ than did the healthy volunteers ($p=0.004$ at week 1, $p=0.04$ at week 2, Figure 11.5). Longitudinal $\text{FeNO}$ trends are examined in more detail below.

**Determinants of $\text{FeNO}$ in adult with pulmonary TB**

Predictors of $\text{FeNO}$ among the 162 AVDAPT study participants at enrolment were evaluated initially in univariate analyses (regression of log-transformed $\text{FeNO}$ on predictor variables). Significant predictors of $\text{FeNO}$ were found to be sex, weight and radiological disease severity. Females had lower median $\text{FeNO}$ than males (13.0 vs 17.3 ppb, $p=0.05$, Figure 11.6). Lower bodyweight (or BMI) predicted lower $\text{FeNO}$ (weight: $p=0.002$, Figure 11.7; BMI: $p=0.02$). More extensive radiological disease also predicted
lower \( \text{FeNO} \) (\( p=0.03 \)). However in multivariate models controlling for sex, age, ethnicity, height and smoking status, only the association with weight remained significant.

Figure 11.5: Exhaled nitric oxide in healthy volunteers and TB patients at week 0 and during TB treatment

*All \( p \) values calculated from 2-sample t tests (log-transformed \( \text{FeNO} \)). Numbers above data indicate median \( \text{FeNO} \) and number of observations at each time point.

Other small differences were detected which failed to achieve statistical significance: Non-Papuans had marginally lower median \( \text{FeNO} \) than Papuans (15.5 vs 16.9 ppb, \( p=0.1 \)) as was observed among healthy volunteers, and \( \text{FeNO} \) was also lower in HIV+ compared with HIV- people (15.6 vs 17.3 ppb, \( p=0.4 \)), and in current smokers compared with non-smokers (15.8 vs 16.4 ppb, \( p=0.8 \)). No associations were evident between height, age or serum ionised calcium level and \( \text{FeNO} \).

In examination of other markers of disease severity, trends towards an association between greater TB severity and lower \( \text{FeNO} \) were noted, but none were statistically significant. Illness duration, symptom tally and SGRQ scores were inversely related to \( \text{FeNO} \), while percentage of predicted FEV\(_1\), 6-minute walk distance and haemoglobin
were directly related to \( \text{FeNO} \) (p>0.05 level in univariate analyses and when controlling for weight or other variables).

**Figure 11.6: Exhaled nitric oxide according to sex in TB patients at week 0**

*\( p=0.05^* \)

<table>
<thead>
<tr>
<th>Sex</th>
<th>Median FeNO</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>17.3</td>
</tr>
<tr>
<td>Female</td>
<td>13.0</td>
</tr>
</tbody>
</table>

*\( p \) value calculated from regression of log-transformed \( \text{FE}_{\text{NO}} \) (dependent variable) on sex (independent variable). Bars indicate median \( \text{FE}_{\text{NO}} \).

**Figure 11.7: Exhaled nitric oxide according to weight in TB patients at week 0**

*\( p=0.002^* \)

<table>
<thead>
<tr>
<th>Weight</th>
<th>FeNO (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt;40kg</td>
<td></td>
</tr>
<tr>
<td>40-49.9kg</td>
<td></td>
</tr>
<tr>
<td>50-59.9kg</td>
<td></td>
</tr>
<tr>
<td>&gt;=60kg</td>
<td></td>
</tr>
</tbody>
</table>

*\( p \) value calculated from regression of log-transformed \( \text{FE}_{\text{NO}} \) (dependent variable) on weight (independent variable). Bars indicate median \( \text{FE}_{\text{NO}} \).

**\( \text{FE}_{\text{NO}} \) TRENDS DURING TREATMENT FOR PULMONARY TB**

As noted above, median \( \text{FE}_{\text{NO}} \) readings initially decreased, reaching a nadir between weeks 2 to 4, then gradually rose throughout the remainder of the 24-week treatment
duration. Using paired t-tests, the FE\textsubscript{NO} fall between week 0 and week 1 was significant (p=0.005, 87 data pairs), as were the differences in FE\textsubscript{NO} between weeks 2 and 24 (p=0.04, 70 pairs) and weeks 4 and 24 (p=0.001, 67 pairs), although not between weeks 0 and 24 (p=0.3, 71 pairs). However, not all individuals followed this pattern, with longitudinal FE\textsubscript{NO} trends among individuals showing some variability (data not shown). Modest, statistically non-significant direct correlations were found between percentage increases in FE\textsubscript{NO} over time and higher composite clinical outcome scores at weeks 8 and 24 (Figure 11.9).

Figure 11.8: Correlations between change in FE\textsubscript{NO} and clinical outcome score at weeks 8 and 24

Composite clinical outcome scores are calculated as described in Chapter 10. Plots show individual data points and line of best fit.

(a) Week 8 
(b) Week 24

Spearman’s Rho: 0.1, p=0.2. 
Spearman’s Rho: 0.2, p=0.096

Associations between serum ionised calcium (potentially indicative of macrophage activation and 1,25(OH)\textsubscript{2}D\textsubscript{3} synthesis) and FE\textsubscript{NO} were examined at baseline and during follow up. At baseline, no significant association was identified between calcium level and FE\textsubscript{NO} in a linear regression model (p=0.1). During follow up, 23 people developed hypercalcaemia. Median FE\textsubscript{NO} among those individuals at the time of hypercalcaemia occurrence was 12.2 ppb, compared with 16.9 ppb in those same individuals at times of normocalcaemia (p=0.4), consistent with the observation that the timing of hypercalcaemia occurrence coincided with the period between weeks 2 and 4 when minimum FE\textsubscript{NO} also occurred. Examining individual trends, synchronous with the development of hypercalcaemia, FE\textsubscript{NO} rose in 6 people, fell or stayed the same in 15, and was unavailable in 2.
11.4 DISCUSSION

This is the largest study to date of \( FE_{NO} \) in patients with pulmonary TB. The findings provide important and unexpected new contributions to the literature regarding the nature of \( FE_{NO} \) responses in people with pulmonary TB, from which a further understanding of local antimycobacterial immune responses can be extrapolated. Interpretation of these findings is contingent on an understanding of \( FE_{NO} \) measurement characteristics in the field, and \( FE_{NO} \) values in locally-recruited healthy volunteers.

These interim results have several limitations. Firstly, although longitudinal findings have been presented here as well as baseline results, the potential impact of the study interventions (especially L-arginine) on \( FE_{NO} \) is readily acknowledged; hence in the absence of randomisation arm information, these results are not intended to provide definitive answers on \( FE_{NO} \) trends over time. Secondly, the methodological issues with regards to use of the NiOX MINO portable analyser \( FE_{NO} \) indicate a need for caution in interpreting the results. Finally, while the sample size is the largest to date investigating \( FE_{NO} \) in TB, full recruitment of TB patients and healthy volunteers will allow greater confidence in interpreting the findings.

\textit{NiOX MINO PERFORMANCE}

NiOX MINO analysers were found to have high repeatability, supporting the manufacturer’s instruction that a single measure is adequate for any given time point. However, \( FE_{NO} \) measured in human biological controls at the field research site was lower when using the portable NiOX MINO analyser than when using the non-portable NiOX FLEX reference analyser. This is in contrast with the findings of Alving et al, who found NiOX MINO readings to be generally slightly \textit{higher} than those obtained from the reference machine.\textsuperscript{103} Additionally, 2 sensors were unstable over time, suffering from significant downward drift. Although other authors have described the need to apply correction factors to account for drift within NiOX MINO sensors over time,\textsuperscript{223} overall the extent of the difference between portable and reference analyser results and the amount of drift seen in some sensors in this study were greater than anticipated.
Environmental conditions (high humidity and ambient temperature) at the field research site are the most likely explanations for these findings. Aerocrine® scientific staff contacted about this issue and an expert adviser in FE\textsubscript{NO} measurement (Dr Cheryl Salome) also agree with this interpretation of the results. These findings clearly establish the need for regulation of ambient conditions when operating NiOX MINO analysers, and are instructive for other researchers seeking to measure FE\textsubscript{NO} in tropical settings. Since differences between the analysers were first recognised, the MINO analysers and their sensors have been stored whenever possible in temperature and humidity-regulated environments. However, during use, the analyser is unavoidably exposed to standard ambient conditions at the non-airconditioned TB clinic (temperature 27 – 34°C, relative humidity 80-99%) (see Figure 4.2, Chapter 4).

Given these findings, appropriate correction factors have been calculated and applied to all FE\textsubscript{NO} values obtained from AVDAPT study participants and healthy controls. Although a systematic difference between the portable and reference analyser might not impact on analysis of trends, application of a correction factor permits the FE\textsubscript{NO} data obtained from TB patients to be compared with the historical controls, and to be compared with the published literature on FE\textsubscript{NO} values obtained at the same flow rate of 50 mL/s.

**FE\textsubscript{NO} in healthy volunteers**

Among volunteers recruited contemporaneously with the AVDAPT study participants, exceptionally high FE\textsubscript{NO} values were measured in some apparently healthy individuals. As discussed in Chapter 3, delineations of a normal reference range for FE\textsubscript{NO} are evolving, but those constructed from studies of Swedish adults suggest that, for people of an age and height comparable to the healthy controls described here, FE\textsubscript{NO} > 25 ppb is considered abnormal.\textsuperscript{105, 223} A possible explanation is that participants labelled as healthy did have underlying unrecognised pulmonary or systemic disease causing elevated FE\textsubscript{NO}. While research assistants are assiduous in ensuring that participants are free of any intercurrent or recent illness including cough in order to satisfy inclusion criteria for eligibility as a healthy control, it is possible that undiagnosed or subclinical asthma or other inflammatory conditions might exist which are unknown to the patient and missed in the screening process. Another possible explanation for the finding of higher-than-expected FE\textsubscript{NO} readings among a supposedly healthy population might
include ethnic or dietary determinants; other authors have proposed that as-yet unidentified factors including nutritional status, genetics and / or undiagnosed or low grade lung inflammation might contribute to inter-subject FE\textsubscript{NO} variability.\textsuperscript{107} Nevertheless, such underlying factors are likely to apply across both control and TB groups in this setting, and highlight why control data derived from the local population are important. Experience with evaluating FE\textsubscript{NO} in this environment in the past is limited to the study by Yeo and colleagues,\textsuperscript{56} the healthy control data from which has been used in the analysis here. This previous study did not examine differences in FE\textsubscript{NO} between healthy Papuans and Non-Papuans. The new data presented here demonstrate that Papuans may have higher FE\textsubscript{NO} than Non-Papuans, both among healthy volunteers and TB patients, although these findings did not reach statistical significance.

Few predictors of FE\textsubscript{NO} in healthy people have been identified in the past: initially, factors such as age and height were not thought to significantly influence FE\textsubscript{NO},\textsuperscript{100} but a more recently-published study of over 1000 Swedish adults who had never smoked was able to show a significant relationship, whereby increasing age and height predicted higher FE\textsubscript{NO} values.\textsuperscript{107} That age and height were not found to significantly predict FE\textsubscript{NO} among the healthy volunteers reported here may therefore reflect the relatively small sample size and the heterogeneous nature of the volunteer population, comprising a mix of ethnicities and smoking status. A potential limitation in interpreting the pooled results of historical and contemporary controls is the different methods used to measure FE\textsubscript{NO} (the reference analyser in the former, and the portable analyser with application of a correction factor in latter). Smoking is one of the few consistently-identified predictors of FE\textsubscript{NO}, being associated with depressed FE\textsubscript{NO}.\textsuperscript{100, 109} Although smokers (both healthy volunteers and AVDAPT patients) did have slightly lower FE\textsubscript{NO} values than non-smokers, the differences were non-significant. Rather than bringing into question the reliability of the FE\textsubscript{NO} assay, this may again be a product of the heterogeneity of the groups studied, and in the case of the volunteers, the relatively small sample size, such that other unidentified influences might have impacted on NO dynamics in the lung, obscuring the significance of the smoking effect.

**FE\textsubscript{NO} IN PEOPLE WITH PULMONARY TB**

A key hypothesis articulated in the Aims of this thesis is that FE\textsubscript{NO} will be increased in participants with pulmonary TB compared with healthy controls, will be inversely related to disease severity at baseline, and will return towards normal by the end of
therapy. The theoretical basis for the generation of this hypothesis included the following principles, unpinned by the clinical findings reported by Wang et al.\textsuperscript{110} (1) in general, \( \text{FE}_{\text{ENO}} \) is increased in pulmonary inflammatory conditions;\textsuperscript{100} (2) specifically, NO as a mediator of anti-mycobacterial immune responses would be expected to be increased in response to infection with TB;\textsuperscript{38, 110, 224-229} (3) an inverse relationship with disease severity would arise due to people with insufficient NO responses being less able to contain MTB proliferation and TB-related pathology.\textsuperscript{38, 230, 231}

In dramatic contrast to the stated hypothesis, the data presented here indicate that \( \text{FE}_{\text{ENO}} \) appears to be no different, or in fact potentially lower (but not statistically significantly), in TB than in health. Why might people with pulmonary TB have non-elevated, or lower, \( \text{FE}_{\text{ENO}} \) than healthy volunteers? As reviewed in the paper reproduced in Chapter 3, NO production from L-arginine by the enzyme NOS2 is a key component of the anti-mycobacterial response. An absence of elevated NO in active pulmonary TB infection could therefore indicate one or more of the following possible hypotheses.

Firstly, an impaired ability to generate adequate NO might be a precursor for the development of active TB, rather than low-normal \( \text{FE}_{\text{ENO}} \) necessarily being a consequence of active TB. Thus, low NO-generating capacity in an individual’s pulmonary tract might be a risk factor for active TB. This concept is lent plausibility by the additional inverse relationship identified between disease severity (chiefly lower weight) and \( \text{FE}_{\text{ENO}} \), a finding which is consistent with our original hypothesis. A defective capacity to generate NO from L-arginine might arise due to L-arginine deficiency. L-arginine deficiency can be due to malnutrition or disease-related L-arginine metabolism, since arginase can be induced by MTB in macrophages, thus diverting L-arginine substrate away from NO production and instead promoting the breakdown of L-arginine to ornithine.\textsuperscript{94} Alternatively, NOS2 production or activity might be impaired because of a cytokine profile favouring diminished activity in the IFN\( \gamma \)-JAK/STAT-1 pathway, resulting in down-regulation of the NOS2 gene and decreased NOS2 generation.\textsuperscript{38} With less NO available in alveolar macrophages and surrounding tissues, MTB proliferation might be less well controlled,\textsuperscript{224, 225, 228} and, extrapolating from animal models, more severe pulmonary pathology might develop.\textsuperscript{38, 230, 231} Alternatively NO quenching from disease-induced superoxide or other radicals may reduce local NO bioavailability in TB. These possibilities strengthen the rationale for trialling adjunctive L-arginine in people with active TB, and further suggest that
maintaining adequate L-arginine levels through optimised nutrition may be an important preventive strategy in people with latent TB infection.

Secondly, the capacity to generate NO might be high, but actual NO generation at the level of the alveolar macrophage could be poorly reflected by the measurement of NO in expired breath after transiting the pulmonary tract. NO is a highly volatile gas with a short half life. It is rapidly removed by pulmonary and upper respiratory tract scavenger molecules for conversion in vivo to nitrite or nitrate. However, given the recognised validity of $F_{ENO}$ measurement, including the known association between NOS2 concentration and $F_{ENO}$, this appears to be a less likely explanation.

Thirdly, it is possible that, in contrast to murine models of TB, NO production may not be as important in the human TB immune response as has been suggested by the in vivo and in vitro studies to date. However the evidence for NO importance is substantial: additional to the large body of literature on the role of NO in animal immune responses to MTB, summarised elsewhere, human pulmonary macrophages have been shown to kill mycobacteria only if they express NOS2, with killing prevented with a NOS inhibitor. Rich and colleagues showed that NO production by alveolar macrophages from healthy humans infected ex vivo with MTB correlates with intracellular growth inhibition of MTB. A number of other researchers have identified NO production by macrophages to be a standard human immunological response to MTB infection. While the evidence is strong that NO production is a usual human protective immune response to TB infection, it is possible that this response is of chief importance at the time of initial exposure to MTB, rather than at the later stage of disease (active TB) assessed in this study. NO production may be higher at earlier stages of active disease, but fall as disease progression leads to reduced L-arginine bioavailability and/or NOS2 activity or increased local quenching of NO. Each of these factors may then exacerbate disease progression.

Aside from obvious risk factors such as HIV infection, why it is that some individuals adequately contain latent TB infection, while others eventually progress to active TB, remains a major research question. The data presented here raise the hypothesis that a failure to generate adequate NO in response to MTB infection may be an example of a characteristic identifying those at increased risk of developing active TB. Furthermore, the more defective the NO response, the worse the TB disease severity may be. This
possibility is supported by the finding that weight / BMI significantly inversely predicted $\text{FE}_{\text{NO}}$ in TB patients but not in healthy controls, suggesting that the association is due to TB disease severity rather than body weight or BMI per se influencing $\text{FE}_{\text{NO}}$. The additional inverse association between radiological disease extent and $\text{FE}_{\text{NO}}$ supports this, as does the fact that $\text{FE}_{\text{NO}}$ was marginally lower (but not significantly so) in those with longer illness duration, more symptoms, worse quality of life scores, and HIV-TB co-infection.

The next question raised by this data is: why might $\text{FE}_{\text{NO}}$ decrease after TB treatment is started, as suggested by the AVDAPT data? As mentioned above, longitudinal trends cannot be interpreted with confidence without the availability of randomisation arm information. However, this finding deserves discussion since it is a reverse of the anticipated trend at least among the 50% of study participants who will have been randomised to receive L-arginine.

An initial worsening of disease severity prior to improvement, due to increased inflammatory activity in response to antibiotic treatment, is a well-recognised phenomenon in TB. These paradoxical reactions are noted to occur in approximately 10% of TB patients without HIV, and up to 43% of HIV-TB co-infected patients commencing antiretroviral therapy (in which case, if meeting the case definition, it is termed immune reconstitution inflammatory syndrome). This phenomenon is also thought to account for the development of hypercalcaemia after TB treatment commencement, as the host’s granulomatous response is restored and macrophage activation via toll-like receptor (TLR) pathways leads to increased $1,25(\text{OH})_2\text{D}_3$ synthesis. Since the trend indicated in data from the AVDAPT study participants is for an initial decrease in $\text{FE}_{\text{NO}}$ prior to a later rise, and a fall in $\text{FE}_{\text{NO}}$ (statistically non-significant) in concert with a rise in serum calcium, macrophage activity during this period might become predominantly skewed towards TLR1/2-MyD88-NFkB pathways and away from the IFN$\gamma$-JAK/STAT-1 pathway (see Figure 2, “L-arginine and Vitamin D: novel adjunctive immunotherapies in tuberculosis”, Chapter 3), thereby resulting in increased $1,25(\text{OH})_2\text{D}_3$ synthesis rather than NOS2 up-regulation. Whether or not this is an immunologically beneficial outcome is unclear.

Since many of the $\text{FE}_{\text{NO}}$ associations identified here were modest, definitive conclusions will be deferred until study completion. In particular, assessment of the longitudinal
data will be more meaningful once study participants can be stratified according to the study medication arm to which they were randomised. The early reduction in $F_{ENO}$ may be more apparent in those not receiving L-arginine supplementation.

**Comparison with previously-published findings**

As described in Chapter 3, the two previously published reports investigating $F_{ENO}$ in TB provided conflicting results. The data presented here agree with elements of both papers (Table 11.4), including the inverse association between $F_{ENO}$ and disease severity identified by Wang et al., and the lower (but statistically non-significant) $F_{ENO}$ in TB infection identified by Idh et al.

**Table 11.4: Summary of salient findings from the present study and previous investigations of exhaled nitric oxide in tuberculosis**

<table>
<thead>
<tr>
<th></th>
<th>AVDAPT study</th>
<th>Wang et al, 1998$^{110}$</th>
<th>Idh et al, 2008$^{111}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TB patients</td>
<td>162</td>
<td>19</td>
<td>95</td>
</tr>
<tr>
<td>Healthy controls</td>
<td>83</td>
<td>14</td>
<td>63</td>
</tr>
<tr>
<td><strong>Method</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F_{ENO}$ assay methodology</td>
<td>NIOX MINO (flow rate 50mL/s, inhalation of NO-free air prior to exhalation) with application of correction factor</td>
<td>Slow exhalation (flow rate ~200 mL/s) through open tube, with a probe sampling NO from the stream of expired breath.</td>
<td>NIOX FLEX (flow rate 50mL/s, omission of inhalation of NO-free air prior to exhalation)</td>
</tr>
<tr>
<td><strong>Results</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F_{ENO}$ in TB versus healthy volunteers</td>
<td>Same (non-significant trend towards lower $F_{ENO}$ in TB)</td>
<td>Higher</td>
<td>Same (non-significant trend towards lower $F_{ENO}$ in TB)</td>
</tr>
<tr>
<td>$F_{ENO}$ in HIV+TB+ versus HIV-TB+</td>
<td>Same (non-significant trend towards lower $F_{ENO}$ in HIV+TB+)</td>
<td>Not evaluated</td>
<td>Same (non-significant trend towards lower $F_{ENO}$ in HIV+TB+)</td>
</tr>
<tr>
<td>Association between $F_{ENO}$ and TB severity</td>
<td>$F_{ENO}$ lower in more severe disease</td>
<td>$F_{ENO}$ lower in more severe disease</td>
<td>$F_{ENO}$ independent of disease severity</td>
</tr>
<tr>
<td>Longitudinal trend</td>
<td>$F_{ENO}$ more closely approximates that of healthy controls by 6 months of treatment (net rise in $F_{ENO}$)</td>
<td>$F_{ENO}$ more closely approximates that of healthy controls by 3 months of treatment (net fall in $F_{ENO}$)</td>
<td>Not evaluated</td>
</tr>
</tbody>
</table>
Wang et al\textsuperscript{110} investigated FE\textsubscript{NO} in 19 people with smear positive pulmonary TB (notably excluding people with poor nutritional status) and 14 non-smoking controls (6 with haemoptysis of unknown cause and 8 healthy volunteers) using a now-obsolete method, whereby participants exhaled slowly at a flow rate of around 200 mL/s through an open tube, with a probe sampling NO from the stream of expired breath. Therefore, FE\textsubscript{NO} values are not directly comparable to results obtained at flow rates of 50 mL/s, but nevertheless among TB patients were in a similar range to results reported here. They found higher FE\textsubscript{NO} in people with newly diagnosed TB (16.2±1.2 ppb) compared with controls (6.5±0.9 ppb), net normalisation of FE\textsubscript{NO} by 3 months of treatment (comprising a decrease in 18 patients, but an increase in one), and an inverse association between FE\textsubscript{NO} and disease severity.\textsuperscript{110} Their finding of higher baseline FE\textsubscript{NO} in contrast to the data presented here might be attributable to the different FE\textsubscript{NO} measurement method employed, the small sample size might have been poorly representative of usual populations of pulmonary TB patients, the ‘healthy’ controls might not have been an ideal comparative group since 6 of them had haemoptysis, or since malnourished TB patients were specifically excluded, this may have ensured the inclusion only of people with replete NO substrate (L-arginine) levels, who were therefore capable of adequate exhaled NO production.

The study by Idh et al,\textsuperscript{111} also in smear positive pulmonary TB, suffered from methodological flaws including that inhalation of NO-free air was omitted prior to FE\textsubscript{NO} measurement, and analyses did not control for anthropometric measures or age. Although the difference in FE\textsubscript{NO} was miniscule between the 36 HIV+/TB+ patients (median FE\textsubscript{NO} 14.2 ppb) and 59 HIV-/TB+ patients (median FE\textsubscript{NO} 14.3 ppb), the authors nevertheless found FE\textsubscript{NO} to be significantly lower in HIV+/TB+ than in healthy controls, but that the difference between HIV-/TB+ (and presumably in the 95 TB patients overall) was not significantly lower than in healthy controls. These trends are essentially similar to those found in the AVDAPT study participants.

11.5 CONCLUSIONS

Despite the recognised anti-mycobacterial role of NO in human macrophages, active pulmonary TB does not appear to be associated with elevated FE\textsubscript{NO} compared with controls in the current study (the largest to date), nor in the only other study\textsuperscript{111} which
employed a comparable method of evaluating $\text{FE}_{\text{NO}}$. An impaired ability to generate adequate NO might be a risk factor for the development of active TB, a consequence of active TB and/or contribute to more severe disease in those who do develop active TB. Thus, individuals at risk for impaired NO production, such as those who are relatively L-arginine deficient, may be individuals at risk for development of active TB. Further clarification of the relationship between $\text{FE}_{\text{NO}}$ and TB at diagnosis and during treatment will be obtained through recruitment of the full AVDAPT and healthy volunteer samples, when serum L-arginine levels are available to include in analyses, and when the randomisation code is revealed.

Results reported here also indicate that researchers seeking to measure $\text{FE}_{\text{NO}}$ in tropical environments using NiOX MINO® analysers need to be mindful of keeping the device in a temperature and humidity-regulated environment, to avoid $\text{FE}_{\text{NO}}$ under-estimation.
12 Results VI: HIV-TB co-infection

This chapter describes HIV-TB co-infection among AVDAPT study participants.

12.1 INTRODUCTION

Evolving response to HIV in Timika

The period of June 2008 - October 2009, during which the data analysed in this thesis was collected, witnessed changes in HIV management in Timika including: (1) evolving regulations regarding who is authorised to offer voluntary counselling and testing (VCT); (2) increased integration of HIV testing into routine TB services; (3) variable CD4 count availability; (4) the launch of a Timika District Health Authority TB-HIV collaborative team of medical and administrative staff, and (5) the opening of a second community hospital in September 2008 adding an extra antiretroviral therapy (ART)-prescribing site.

Timika had a fledgling VCT system in place at the commencement of the study in June 2008, and limited access to ART. Traditional VCT comprised an emphasis on pre-test counselling, on the basis that HIV had limited treatment options and a very poor prognosis. The ‘provider-initiated, opt-out’ HIV testing approach aims to increase the focus on treatment, and hence make testing more routine, with greater emphasis on ‘opting out’ (i.e. HIV testing undertaken as part of the diagnostic work-up unless specifically declined), and a focus on post-test rather than pre-test counselling, as for most other serious medical conditions. Provider-initiated opt-out HIV testing is increasingly being deployed internationally, along with wide advocacy for this strategy to improve HIV detection rates. In contrast, rising HIV awareness in Timika has led to the introduction of a stringent VCT process in early to mid 2008. This involves counselling only from a trained counsellor (either a doctor or other health professional with specific VCT certification) and written consent.
In order to centralise HIV care, the District Health Authority, under instruction from national health authorities, has determined that ART be prescribed by designated prescribers at nominated sites only. Thus ART cannot be prescribed at the Timika TB clinic or the adjoining Timika sexual health clinic (although VCT can be provided at both sites). Patients must be referred for ART to one of the 2 district hospitals, where a doctor at each site has authority to prescribe ART. Additionally, a non-government organisation (International SOS) provides ART to employees of Freeport Mining Corporation and their families. Use of isoniazid preventive therapy, acknowledged as an important and underutilised strategy for preventing TB in people living with HIV/AIDS,\textsuperscript{239-241} is not currently routine in Indonesia, including Timika.

**TB-HIV COINFECTION OVERVIEW**

Tuberculosis (TB) and HIV infections are major global health threats. An estimated 1.4 million new TB cases in HIV-positive individuals were reported in 2007,\textsuperscript{2} posing wide-ranging diagnostic, management and economic challenges. HIV confers the greatest risk for TB, increasing the risk of latent TB reactivation 20-fold \textsuperscript{242, 243}. TB is a leading cause of death among people with HIV,\textsuperscript{244} and TB patients co-infected with HIV (HIV+/TB+) have significantly worse outcomes than those without HIV (HIV-/TB+).\textsuperscript{245}

Priorities in addressing TB-HIV co-infection include instituting the World Health Organisation (WHO)’s ‘3Is’ (Intensified TB case-finding, Isoniazid preventive therapy and Infection control),\textsuperscript{246} ensuring routine ascertainment of HIV status in people with TB,\textsuperscript{35} close integration of TB and HIV services,\textsuperscript{235} and universal access to antiretroviral therapy (ART) in HIV-positive people,\textsuperscript{247} including early ART initiation in TB-HIV co-infection.\textsuperscript{8, 235, 248, 249} Acknowledging the improved outcomes achieved with ART,\textsuperscript{249} WHO guidelines have moved beyond 2009 recommendations guided by CD4 count\textsuperscript{235} to now recommend universal ART in people with TB-HIV co-infection.\textsuperscript{248}

Despite these rapid advances in knowledge, substantial barriers persist to the achievement of optimised TB-HIV management goals, especially in lower-income countries. In tackling the challenge of HIV in people with TB, multifactorial barriers include failures to implement HIV testing, failure to prescribe ART or other elements of HIV care, pre-treatment loss to care and post-ART-initiation loss to follow up.\textsuperscript{250}
In Indonesia, HIV infection rates in TB are not routinely reported. A 2006 study utilising unlinked, anonymous testing in people with TB in Yogyakarta found an HIV seroprevalence rate among TB patients of 1.9%. The overall national estimate is 3%. Papua Province has long been recognised as having one of the highest HIV burdens in Indonesia. An ‘Integrated Bio-Behavioral Surveillance’ study in 2006, of 6305 Papua Province residents aged 15-49 years, reported population HIV seroprevalence as 2.4%. This survey also revealed low levels of health knowledge: 51.8% overall had heard of HIV/AIDS (26.3% among those with no or limited education). Only 35.4% knew that condom use was protective. Especially high HIV rates of 26% have been documented in Papuan female commercial sex workers.

Despite Indonesia having a policy to provide free ART since 2003, national capacity to widely roll out HIV care is limited. HIV VCT was taken up by just 4% of TB patients in one 2006 Indonesian study. Recent estimates of ART accessibility indicate that only about 24% of Indonesian people with advanced HIV infection receive ART; this figure is as low as 3% in Papua Province.

The objective of this chapter is to describe current TB-HIV epidemiology and management in Timika in order to tailor future interventions. Specifically, I aim to investigate the burden of HIV infection among adults with smear-positive pulmonary TB, to examine changes over time, to describe current HIV management and its relationship to WHO guidelines, and investigate TB treatment outcomes among TB-HIV co-infected people.

### 12.2 METHODS

Recruitment of study participants and statistical analyses are as described in preceding chapters. VCT is conducted by the TB clinic doctor, one of the medically-trained study Research Assistants, or a trained counsellor at the sexual health clinic which is adjacent to the TB clinic. It is done privately and confidentially, or with a spouse or family member if requested by the patient, or with a parent/guardian if the patient is aged <18 years. An Indonesian national VCT form is used. Detailed education about HIV/AIDS is provided; the patient may decline testing, or demonstrate their agreement by signing their name or applying their thumbprint to the form. The process takes around 30 to 60 minutes.
HIV antibody testing is performed using SD BioLine HIV-1/2 3.0\textsuperscript{TM} antibody test (Standard Diagnostics, Inc). If positive, confirmation is performed using Abbott Determine\textsuperscript{TM} HIV-1/2 (Inverness Medical), and Oncoprobe\textsuperscript{TM} (PT Oncoprobe Utama) point-of-care tests. Similar strategies for HIV testing (i.e. reliance on follow-up point-of-care tests to confirm positive results) have been shown to be valid elsewhere.\textsuperscript{256} SD BioLine HIV sensitivity is reported to be 100%, and specificity to be 99.8%, and for Determine, to be 100% and 99.6% respectively.\textsuperscript{136, 256} In instances where SD BioLine assays have been unavailable, FOKUS\textsuperscript{TM} point-of-care HIV antibody test kits have been used. If there is discordance between tests, the result is reported as ‘intermediate’ and is repeated on a second blood sample within 4 weeks. A fluorescence-activated cell sorter for performing CD4 T cell assays was intermittently available for use two mornings per week at the District Health Clinic laboratory. Its maintenance and operation is independent of the AVDAPT study, and measurement of CD4 count is not included in the study protocol.

WHO 2009 guidelines were used as the best-practice reference, recommending ART initiation within 2-8 weeks after TB treatment commencement for CD4 <200 or unknown, after 8 weeks if CD4 200-350, and deferral of ART if CD4>350.\textsuperscript{235}

\textbf{12.3 RESULTS}

As described in previous chapters, 162 smear-positive pulmonary TB patients were recruited (Table 12.1). One hundred and forty one (87.0%) were offered VCT. Uptake of HIV testing was high (138/141 = 97.9%); only 3 participants (all females, 1 Papuan and 2 Non-Papuan) declined an HIV test. Eighteen of 138 people (13.0%) who had an HIV antibody test were HIV positive, confirmed with 3 rapid assays. Papuans were significantly more likely to be HIV positive than Non-Papuans (14/67=20.9% versus 4/71=5.6%, p=0.01). Papuan women comprised the highest-risk subgroup, although confidence intervals were wide (Figure 12.1). HIV+/TB+ participants were less likely than HIV-/TB+ to be employed (6/18 = 33.3% vs 67/114 = 58.8%, p=0.04, \(\chi^2\) test), and less likely to have achieved an educational level above primary school (10/18=55.6% vs 89/119=74.8%, p=0.05). However, these associations became non-statistically significant when controlling for ethnicity in multivariate logistic regression analyses, since Papuan ethnicity was associated with lower employment and education. HIV status was unrelated to age.
Table 12.1: Characteristics of 138 study participants with known HIV status

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>HIV+</th>
<th>HIV-</th>
<th>p value (HIV+ vs HIV-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number (%)</td>
<td>138</td>
<td>18 (13.0)</td>
<td>120 (87.0)</td>
<td></td>
</tr>
<tr>
<td>Age in years: median (range)</td>
<td>27 (15-65)</td>
<td>31 (16-60)</td>
<td>27 (15-56)</td>
<td>1.0</td>
</tr>
<tr>
<td>Papuan: no. (%)</td>
<td>67 (48.6)</td>
<td>14 (77.8)</td>
<td>53 (44.2)</td>
<td>0.01</td>
</tr>
<tr>
<td>Female: no. (%)</td>
<td>43 (31.2)</td>
<td>7 (38.9)</td>
<td>36 (30.0)</td>
<td>0.4</td>
</tr>
<tr>
<td>Current smoker: no. (%)</td>
<td>42 (30.4)</td>
<td>5 (27.8)</td>
<td>37 (30.8)</td>
<td>0.8</td>
</tr>
<tr>
<td>Highest educational attainment: no. (%)</td>
<td>10 (7.2)</td>
<td>4 (22.2)</td>
<td>6 (5.0)</td>
<td>0.05*</td>
</tr>
<tr>
<td>No schooling</td>
<td>28 (20.3)</td>
<td>4 (22.2)</td>
<td>24 (20)</td>
<td></td>
</tr>
<tr>
<td>Primary school</td>
<td>96 (69.6)</td>
<td>10 (55.6)</td>
<td>86 (71.7)</td>
<td></td>
</tr>
<tr>
<td>High school</td>
<td>4 (2.9)</td>
<td>0 (0)</td>
<td>4 (3.3)</td>
<td></td>
</tr>
<tr>
<td>Academy or university</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Unemployed: no. (%)</td>
<td>59 (44.7)</td>
<td>12 (66.7)</td>
<td>47 (41.2)</td>
<td>0.04*</td>
</tr>
<tr>
<td>Owns telephone: no. (%)</td>
<td>77 (55.8)</td>
<td>8 (44.4)</td>
<td>69 (57.7)</td>
<td>0.3</td>
</tr>
</tbody>
</table>

**Clinical and laboratory investigations**

<table>
<thead>
<tr>
<th></th>
<th>All</th>
<th>HIV+</th>
<th>HIV-</th>
<th>p value (HIV+ vs HIV-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMI in kg/m²: mean (range)</td>
<td>19.3 (12.9-32.5)</td>
<td>19.2 (12.9-26.7)</td>
<td>19.4 (13.3-32.5)</td>
<td>0.8</td>
</tr>
<tr>
<td>Percentage of predicted FEV₁: mean (range)</td>
<td>63.9 (16.6-108.5)</td>
<td>59.5 (16.5-92.0)</td>
<td>64.6 (23.9-108.5)</td>
<td>0.3</td>
</tr>
<tr>
<td>6 minute walk test in m: median (range)</td>
<td>415 (0-612)</td>
<td>390 (0-485)</td>
<td>415 (75-612)</td>
<td>0.04</td>
</tr>
<tr>
<td>St George’s Respiratory Questionnaire† total score: median (range)</td>
<td>38.3 (5.2-91.9)</td>
<td>41.7 (13.6-67.0)</td>
<td>38.1 (5.2-91.9)</td>
<td>0.4</td>
</tr>
<tr>
<td>Haemoglobin in g/dL: mean (range)</td>
<td>12.4 (7.1-16.0)</td>
<td>11.2 (8.5-12.9)</td>
<td>12.6 (7.1-16.0)</td>
<td>0.002</td>
</tr>
<tr>
<td>White cell count x 10⁹/L: mean (range)</td>
<td>8.0 (1.6-22.7)</td>
<td>6.8 (2.4-13.2)</td>
<td>9.0 (1.6-22.7)</td>
<td>0.01</td>
</tr>
<tr>
<td>Sputum smear ≥2+: no. (%)</td>
<td>61 (45.2)</td>
<td>5 (27.8)</td>
<td>56 (47.9)</td>
<td>0.1</td>
</tr>
<tr>
<td>Chest X-ray</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total score: median (IQR)</td>
<td>67 (4-140)</td>
<td>57 (19-121)</td>
<td>68 (6-140)</td>
<td>0.5</td>
</tr>
<tr>
<td>Cavitary disease on CXR: no. (%)</td>
<td>74 (55.2)</td>
<td>6 (33.3)</td>
<td>68 (58.6)</td>
<td>0.04</td>
</tr>
<tr>
<td>Pleural effusion: no. (%)</td>
<td>23 (17.2)</td>
<td>7 (38.9)</td>
<td>16 (13.8)</td>
<td>0.009</td>
</tr>
</tbody>
</table>

*Differences in education and employment were not significant in multivariate models controlling for ethnicity. †St George’s Respiratory Questionnaire results were only available in 119 participants (15 HIV+ and 104 HIV-)*
**LONGITUDINAL TREND IN TB-HIV CO-INFECTION RATE**

In a previous study of smear-positive pulmonary TB patients at the same Timika TB clinic (2003-2004), HIV status was ascertained in all study participants; 5/112 (4.5%) were HIV positive, all Papuan. Compared with these earlier rates, data presented here demonstrate a significant rise in TB-HIV co-infection in Timika in the last 4 years (p=0.03, Figure 12.2).

**CLINICAL CHARACTERISTICS**

Neither weight nor BMI differed among HIV+/TB+ and HIV-/TB+ study participants, including when controlling for ethnicity and sex (Table 12.1). Exercise tolerance (6MWT) was significantly lower in HIV+/TB+ compared with HIV-/TB+, as was haemoglobin (p=0.002) and total white cell count (p=0.01). These associations remained significant when controlling for weight, ethnicity and sex. Mean pulmonary function results were in the moderately impaired category overall and in the HIV-/TB+ group (i.e. FEV$_1$ 60-69% of predicted), but in the moderate-severely impaired category (i.e. FEV$_1$ 50-59% of predicted) in the HIV+/TB+ group, but this difference was not
statistically significant (p=0.3, Table 12.1). Quality of life scores (SGRQ) did not significantly differ according to HIV status (Table 12.1). No significant differences were found in the rates of reported symptoms at TB diagnosis (including fever, malaise, nausea, vomiting, diarrhoea, dyspnoea, chest pain, haemoptysis, rash etc).

Chest x-ray findings (Table 12.1) differed significantly in TB-HIV co-infected patients, with cavitary disease being less common (p=0.05), and pleural effusion (i.e. extrapulmonary TB) being more common (p=0.009), although overall extent of radiological disease (chest x-ray score) did not differ (p=0.6). TB-HIV co-infected patients also had lower bacillary load in sputum, but this difference was not statistically significant (Table 12.1).

**Figure 12.2: HIV-TB co-infection rates in Timika in 2003-4 compared with 2008-9**

![HIV-TB co-infection rates in Timika in 2003-4 compared with 2008-9](image)

*p value calculated using Fisher’s exact test

**Outcome**

HIV status did not significantly influence time to sputum smear conversion (hazard ratio [HR] 0.7, 95% CI 0.41 - 1.3, p=0.2; see also Chapter 10). The HIV+/TB+ group had a similar overall likelihood of treatment success (HIV+/TB+ cure or completion: 85.7%; HIV-/TB+ cure or completion: 92.6%, p=0.2; Table 12.2). However, they were more likely to suffer an serious adverse event, although numbers were very small (Table
12.2). These comprised in the HIV-/TB+ group: 2 deaths (1 progressive respiratory illness due to TB or secondary pneumonia, and 1 stroke complicated by aspiration pneumonia) and 1 hospitalisation (due to pneumothorax and severe malnutrition, BMI 12.9 kg/m\(^2\)), and in the HIV-/TB+ group, no deaths and 2 hospitalisations (1 vomiting / dehydration and 1 multi-drug resistant-TB with progressive pulmonary disease and large pleural effusion). The relationship between HIV status and default rates could not be determined, since people who defaulted often did so prior to HIV testing being offered (of the original 162 study participants, 8 defaulted: of these 3 were HIV negative, none were known to be positive, and 5 had unknown status).

Table 12.2: Treatment outcome in HIV positive and HIV negative study participants
N=96 (known HIV status and 6 months follow up competed)

<table>
<thead>
<tr>
<th>Outcome</th>
<th>HIV positive</th>
<th>HIV negative</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>14</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>Cure</td>
<td>11 (78.6)</td>
<td>65 (79.3)</td>
<td>0.2</td>
</tr>
<tr>
<td>Complete</td>
<td>1 (7.1)</td>
<td>10 (12.2)</td>
<td></td>
</tr>
<tr>
<td>Failed</td>
<td>0 (0)</td>
<td>1 (1.2)</td>
<td></td>
</tr>
<tr>
<td>Default</td>
<td>0 (0)</td>
<td>3 (3.7)</td>
<td></td>
</tr>
<tr>
<td>Died</td>
<td>2 (14.3)</td>
<td>0 (0)</td>
<td></td>
</tr>
<tr>
<td>Transferred</td>
<td>0 (0)</td>
<td>3 (3.7)</td>
<td></td>
</tr>
<tr>
<td>Serious adverse event: no. (%)</td>
<td>3/18 (16.7)</td>
<td>2/120 (1.7%)</td>
<td>0.02</td>
</tr>
</tbody>
</table>

**HIV MANAGEMENT**

CD4 counts were obtained in 6 patients (Table 12.2). According to 2009 WHO guidelines,\(^{235}\) 14 people were eligible for early ART initiation (CD4<200 cells/\(\mu\)L or unknown), 1 for ART initiation after week 8 (CD4 200-350), and 3 for deferred ART initiation (CD4>350). However, during their 6-month TB treatment period, only one person with TB-HIV co-infection was successfully commenced and maintained on ART, 1 patient with known HIV at the time of TB diagnosis was already taking and remained on ART throughout her involvement in the study, and 2 additional patients who had been referred for ART were commenced by the authorised ART prescriber after their TB treatment was completed.
Table 12.3: TB-HIV coinfection management

<table>
<thead>
<tr>
<th>CD4+ T-cell count: no. (%)</th>
<th>TB-HIV co-infected study participants</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+ T-cells/μL: median (range)*</td>
<td>6 (33)</td>
</tr>
<tr>
<td>CD4+ T-cells &lt;200</td>
<td>318 (18-739)</td>
</tr>
<tr>
<td>CD4+ T-cells 200-350</td>
<td>2</td>
</tr>
<tr>
<td>CD4+ T-cells &gt;350</td>
<td>1</td>
</tr>
<tr>
<td>CD4+ T-cells unknown</td>
<td>3</td>
</tr>
<tr>
<td>Anti-retroviral therapy: no. (%)</td>
<td>6 (33)</td>
</tr>
<tr>
<td>Commenced prior to TB diagnosis</td>
<td>4/18 (22)</td>
</tr>
<tr>
<td>Commenced during TB treatment</td>
<td>1</td>
</tr>
<tr>
<td>Commenced after TB treatment completed</td>
<td>1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>ART type</th>
<th>Co-trimoxazole: no. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AZT / 3TC / nevirapine</td>
<td>11/18 (61)</td>
</tr>
<tr>
<td>AZT / 3TC / efavirenz</td>
<td>1</td>
</tr>
</tbody>
</table>


12.4 DISCUSSION

**TB-HIV co-infection rates**

This study has identified that TB-HIV co-infection rates have significantly risen during 5 years in Timika, Indonesia. Among the Indigenous Papuan subgroup, HIV seroprevalence in TB patients rose from 4.5 to 20.9%. This remains lower than in some global regions; for example, the estimate for the WHO Africa region is 51%. However, it is among the highest reported from Asia, and the rate of change has been rapid, outstripping local capacity to adequately respond to the crisis. HIV co-infection rates differ markedly across Asia, ranging from 0.5% in a large study in Guangxi, China, to an estimated 3% overall for Indonesia and 17% in Thailand. Few published data are available for comparison from neighbouring Papua New Guinea, but the 2009 WHO report indicates 14.5%.

**Differences between HIV positive and negative TB patients**

Reasons for ethnic differences in HIV rates were not investigated in this study but may comprise a combination of factors including sexual behaviours, knowledge of sexual
health and transmission prevention, and possibly, the difference in male circumcision rates (about 5% of Papuans and 70% of Non-Papuans are circumcised).\textsuperscript{124} Injecting drug use is not thought to contribute to HIV transmission in Papua Province.\textsuperscript{124} The trend towards women being at greater risk in Timika is in keeping with findings elsewhere.\textsuperscript{258} HIV+ people were less likely to be educated or employed, indicating that HIV education is need at the community level, not just targeting schools or workplaces.

Clinical differences according to HIV status in this study were not large. A limitation of the study is that the relatively small numbers mean it may be under-powered to adequately detect differences between HIV+ and HIV- groups. No significant differences in symptoms or perceived health-related quality of life were identified. Weight (or BMI) was also not different at baseline. This may indicate that HIV infections overall were not advanced, also supported by the relatively well-preserved CD4+ T-cell counts in some individuals (Table 2). These findings emphasise the importance of routine HIV testing in TB rather than restricting testing to those with additional features suspicious for HIV (such as oesophageal candidiasis), as has been practiced elsewhere.\textsuperscript{259} The HIV+/TB+ group did have significantly reduced exercise tolerance. Haemoglobin was also significantly lower in those with HIV; contributors to this are likely to be multifactorial in Timika, which is characterised by high rates of \textit{Plasmodium falciparum} and \textit{vivax} malaria.\textsuperscript{117} Insufficient dietary iron intake is also likely to be common. This illustrates the compounding effect of overlapping endemic illnesses, potentially contributing to other poor health outcomes such as the very high maternal and infant mortality in Timika.\textsuperscript{127} People with HIV+/TB+ status are known to have higher mortality rates than those who are HIV-/TB+,\textsuperscript{245} reinforced by present findings.

Lower rates of cavitary disease and higher likelihood of pleural effusion were identified among HIV positive people at TB diagnosis in this study. These findings are consistent with previous studies examining chest x-rays in TB-HIV co-infection\textsuperscript{260, 261} Since cavitation largely determines sputum bacillary grade,\textsuperscript{191, 217} TB-HIV co-infection is also associated with higher likelihood of smear-negative disease, or low bacillary density in smear-positive disease, in proportion to CD4 count.\textsuperscript{262} Our data support these findings, although not reaching statistical significance. Such findings emphasise the difficulty of establishing TB diagnoses (based on sputum smear and radiological appearance) in HIV infection. Problems in confidently excluding active TB in HIV contribute to some
persisting reluctance to widely roll-out isoniazid preventive treatment, an important and underutilised strategy for preventing TB in people with HIV/AIDS. Indeed, this strategy is not currently routine in Indonesia. Improving diagnostic sensitivity through inexpensive measures in laboratories (e.g. sputum concentration prior to ZN staining, fluorescence microscopy using an inexpensive light-emitting diode light source, or simple culture-based techniques) and clinics (e.g. educating medical staff to appreciate radiological pathology characteristic of TB-HIV co-infection), are not necessarily beyond the means of low-income settings.

**BARRIERS TO HIV TESTING AND TREATMENT**

In this study, HIV status was established in 138/162 (85.2%) participants. The refusal rate was low. Missed opportunities for HIV diagnosis are widely recognised internationally as well as in Timika. For example, the HIV status of only 35% of Australian TB cases was ascertained in 2006, and nearly half of all TB patients in London in 2003-4 were not offered HIV testing. Barriers to HIV testing in the current study relate primarily to availability of a certified counsellor, availability of a private room, and the time required to provide pre-test counselling. Conducting VCT in a confidential manner is difficult in a crowded TB clinic, reinforcing previous findings of the requirement for the right structural conditions to be present at a clinic to support effective VCT. Given the lack of general knowledge about HIV, reasonably high pre-test probability of a positive result (especially among Papuans), and the degree of stigmatisation suffered by HIV positive individuals, the pre-test counselling process in Timika is justifiably lengthy. On identifying HIV positivity, access to appropriate care including ART and condoms is limited; thus the benefit of HIV status knowledge is reduced. Therefore prevailing attitudes among local medical staff has been to take a cautious approach, even if this leads to incomplete ascertainment of HIV status.

Deployment of provider-initiated opt-out HIV testing has been widely advocated as a strategy to improve HIV detection rates. However, a stringent VCT process was introduced in Timika in 2008, stipulating the requirement for written consent, and for only a doctor or professional with specific certification, to conduct VCT. A study investigating barriers to HIV testing among TB patients in another Indonesian province identified both patient and health provider factors as reasons for
low VCT uptake. Among patients, these included low HIV knowledge, the disincentive of having to access and endure VCT, and fear of knowing the test results; and among health care providers: low HIV knowledge, communication issues, concern about patients feeling offended, stigmatization and additional work load.

Access to CD4+ T-cell testing was limited due to difficulties in maintaining the analyser and lack of staff familiarity with its operation. Only four (5.6%) of the HIV positive study participants successfully commenced ART, 2 after TB treatment completion, even though 15 were eligible for ART during TB treatment according to 2009 guidelines. WHO guidelines on the timing of ART initiation in TB have evolved rapidly on the basis of new trials, such that universal ART initiation regardless of CD4 count is now advocated in TB-HIV co-infection. Integration of TB-HIV is strongly advocated. However, a chief barrier to ART initiation in Timika is the absence of such integration, with no authorised ART-prescriber available in the vicinity of the TB clinic. In Indonesia, ART can be prescribed only by a designated person at nominated sites (usually hospitals). Even if a patient is successfully referred to, and attends, a designated ART-prescription site, reasons for low ART prescription rates cited by medical staff include concern about potential poor adherence, and drug toxicities or paradoxical reactions. Education of healthcare providers about ways to support adherence and manage TB-HIV co-infection is therefore greatly needed.

12.5 CONCLUSION

Rising HIV rates in Timika are a serious concern. Timika has experienced large recent population fluxes related to the local mining industry attracting migrants from rural areas and other parts of Indonesia, and thus presents a classic demographic scenario for burgeoning HIV rates. The association between HIV and migrant labourers, especially mine workers and a thriving commercial sex industry, has been well established elsewhere.

These data provide an important South Asian perspective on the overlapping TB and HIV epidemics, and illustrate the added morbidity suffered by HIV+ compared with HIV- individuals with TB. This study demonstrates that, despite knowledge of TB-HIV management guidelines and even within the setting of a well-regarded TB Directly
Observed Treatment facility, management of TB-HIV co-infection poses an enormous challenge in resource-limited environments. Multiple interventions are now required at community, patient and healthcare provider levels, to address rising HIV rates and the barriers to testing and treatment.

Some improvements have been effected by AVDAPT study investigators through the provision of up-to-date evidence, especially WHO guidelines, to local practitioners and authorities; in particular, we have enabled VCT to be conducted at the TB clinic and by AVDAPT research assistants, and promoted early referral for ART. Data arising from this study will continue to be regularly presented to the District Health Authority to assist in guiding the local response to HIV-TB management. The recent publication of revised WHO guidelines on TB-HIV management provides a timely opportunity to incorporate these into national guidelines, and support their implementation in community settings.

**Figure 12.3:** Meeting between AVDAPT research personnel and local HIV care providers for discussion of HIV-TB management in Timika

*Foreground (L to R): Dr Enny Malonda (RSMM), Dr Andri Wiguna (Timika TB clinic), Dr Dina Bisara Lolong (NIHRD), Dr Enny Kenangalem (NIHRD-MSHR Research Program and Dinas Kesehatan).*

*Photo: Anna Ralph*
Chapter 13: Conclusion

13 Conclusions and Future Directions

“Tuberculosis is arguably among the most written about diseases in the history of medicine. It reaches far into the past, as there was clear evidence of its existence 15000 years ago, and has received the attention of the Old Testament, Hipocrates, and Gallen. One would not have foreseen, however, that as we approach 2012—the 130th anniversary of Robert Koch’s conclusive demonstration of the bacterial cause of tuberculosis—the disease would remain prominent in medical literature.”

TB is a disease of exceptional significance to individuals and to global public health. Major recent progress has been achieved in the collective effort to decrease TB rates. A key challenge is to implement these developments in parts of the globe that most need them. The mechanism by which AVDAPT researchers have sought to contribute to this process is through bringing research of an internationally-recognised standard, with associated potential to build capacity and assist in elevating local standards of care, to a high TB-burden area.

This thesis demonstrates both barriers and solutions to research implementation in such a setting. A notable barrier is evident in the omission from current analyses of blood results requiring off-shore processing. On the other hand, effective solutions to other barriers are evident, such as the use of inexpensive, locally-available tests (e.g. 6-minute walk test) where possible, and the use of an in-country laboratory as soon as it became accredited, to achieve the primary trial endpoint of sputum culture. Other creative approaches have been required to address problems familiar in high TB-burden settings, such as intermittent electricity supplies, the impact of environmental conditions on equipment, transportation problems (for people, consumables and sputum samples) and staff availability and training. The successful recruitment, randomisation, evaluation and follow up to date of 162 study participants, with less than anticipated losses to follow up, testifies to the ability to conduct complex trials in Timika despite these challenges.

Conclusions of the thesis can be drawn firstly from findings pertaining to epidemiological, clinical and immunological characteristics of people with pulmonary
TB in Timika, and secondly, from findings relevant to the methodological development and operation of the AVDAPT clinical trial, as discussed below.

13.1 CLINICAL FINDINGS

A major contribution of this thesis relates to the investigation of exhaled nitric oxide in TB. $F_{ENO}$ was not elevated in TB patients compared with healthy volunteers, and was lower still in worse disease. These findings suggest that an impaired ability to generate adequate NO (e.g. due to absolute or relative L-arginine deficiency) might therefore contribute to the host’s inability to adequately contain infection with MTB. Also, as hypothesised, demonstration of an inverse relationship between baseline pulmonary NO production and disease severity provides supportive data for a disease protective effect of NO (and hence L-arginine) in TB. These findings therefore support the rationale for conducting a trial of adjunctive L-arginine in TB.

A number of further original findings were identified in cross-sectional analyses. The standard malnutrition definition (BMI<18.5kg/m$^2$) appeared to underestimate malnutrition in Papuans, especially Papuan males. While this finding is consistent with recognised typical Melanesian body habitus, no existing literature has been identified which remarks upon this. This indicates a need to further investigate and revise, if necessary, this definition in Melanesian people, to avoid under-diagnosis of malnutrition. In the AVDAPT study, BMI will not be relied on as a nutritional marker: the key nutrients of interest are L-arginine and vitamin D, and an individual’s nutritional status with regards to these molecules will be defined according to serum levels, when available.

The reasons for male over-representation among TB cases need to be identified in different geographical settings, in order to ensure that appropriate TB prevention and care is reaching all parts of the population. In Timika, higher TB rates in men appear to be due to higher actual rates of TB, rather than higher rates of smear positivity or health care attendance, among males.

Another previously unreported finding arising from this data analysis is that TB patients with more evidence of malnutrition, according to lower body weight and haemoglobin, had worse pulmonary function (adjusting for height, sex and ethnicity). This raises
mechanistic questions of whether the increased caloric demands associated with tachypnoea and dyspnoea in TB might contribute to weight loss, or whether immunological consequences of malnutrition could impair lung reparative responses to tissue damage. Studies of nutritional supplementation in TB to date, summarised in Chapter 3, have mainly examined anthropometric and bacteriological outcomes. This new finding suggests that respiratory outcome measures ought to be included in such nutrition intervention studies, as will be the case in the AVDAPT study.

Exercise tolerance was found to be related to symptoms and illness duration rather than pulmonary function in TB. This measure therefore provides a useful additional means of assessing the systemic impacts of TB, beyond respiratory disability alone. It provides insights into TB-related burden of disease, specifically, the difficulty of achieving exercise-dependent activities including walking to the clinic for treatment, and engaging in employment. St George’s Respiratory Questionnaire results were found to accurately capture people’s perceived quality of life in active TB, correlating significantly with symptoms, exercise tolerance and lung function. These findings indicate that this essentially cost-free test therefore provides a further valuable tool with which disease burden and response to treatment can be measured. Sputum AFB grade, although significantly related to radiological disease extent and weight, was not significantly associated with other test results. This finding supports the use in the AVDAPT study of a range of measures to provide a comprehensive assessment of TB severity.

In the current era in which increasing numbers of TB treatment trials are needed in drug-susceptible TB, MDR-TB and HIV-TB co-infection, increasingly sophisticated outcome assessments are being sought, ranging from surrogate biomarkers (which might predict cure or relapse), to quality of life measures (which can provide insight into clinical and socioeconomic disease impacts). The findings reported here contribute important data on outcome assessments which grade different aspects of TB severity and are suitable for use in high TB-burden countries. Further, these results contribute to the understanding of TB pathology and its clinical consequences, and indicate specific areas needing further investigation.
13.2 HIV-TB AND MDR-TB TRENDS

Compared with many other high TB-burden settings, Timika has relatively low MDR-TB and HIV-TB co-infection rates. Nevertheless, major challenges in health care delivery have arisen in Timika, as the rapid rise in HIV-TB co-infection has outstripped local resources and expertise. Implementation of WHO guidelines on integration of HIV and TB services, and improvements in comprehensive HIV care including access to antiretroviral therapy, will be a major focus of future work in Timika. In keeping with international trends, data from AVDAPT study participants indicate that the Indigenous population in Papua Province suffers poorer socio-economic and health status than the non-Indigenous population; this disparity is most particularly evident in their respective HIV-TB co-infection rates.

MDR-TB rates among new TB cases have remained unchanged over 5 years in Timika, indicating that transmitted MDR-TB remains fortunately uncommon relative to the dominant circulating drug-susceptible strains. Reasons for this are unclear, given the presence of a suitable recipe for rising MDR-TB rates (lack of available MDR-TB treatment and rising HIV rates). A potential rise in MDR-TB may therefore be lagging behind that of HIV, and hence continued monitoring and vigilance, and introduction of appropriate treatment regimens, are major local public health priorities. Data cannot be provided here on acquired resistance rates, since the study protocol excludes relapsed TB cases from enrolment. During the course of the study, the establishment of links with a reference laboratory capable of culture and drug susceptibility testing has increased the options for local practitioners to seek results for their patients with suspected MDR-TB, despite them not being enrolled in the study. This has identified at least one further case of MDR-TB in a re-treatment patient at the study site.

13.3 CONCLUSIONS FROM THE AVDAPT STUDY TO DATE

In this thesis I have confirmed the feasibility of performing the measures described in the study protocol, identified the relationships between these, and refined study endpoints, especially the composite clinical outcome score and the radiological score. A suitable composite clinical outcome score, which appears to be clinically valuable and adequately discriminatory in the current dataset, was devised. Radiological improvement was selected as a secondary endpoint, but literature review demonstrated a
The surprising lack of standardisation in this field, prompting the investigation of ways to apply a single numerical score to an x-ray. The two scoring methods thus developed (and, in the case of the x-ray score, validated in a second dataset) for use in the AVDAPT study are likely to also have wider applicability, with potential utility for other TB clinical trialists.

Examination of adverse events in study participants indicated no safety concerns for participants randomised to adjunctive therapy were identified; specifically, the hypercalcaemia data corroborate mounting evidence for vitamin D safety in active TB, despite some persisting misconceptions on this issue lingering in contemporary literature. An additional insight provided by the analysis of adverse events was the high morbidity among TB patients (with half in this study reporting new onset of gastrointestinal disturbance during the first 8 weeks of treatment), which may be overlooked in standard practice when patients are not assiduously questioned about such symptoms on a weekly basis.

The importance of using a locally-derived reference range for exercise tolerance, for comparison with results in AVDAPT study participants, was illustrated by the finding of short 6-minute walk distances among healthy Timika volunteers relative to volunteers in other international settings (not unexpected, given short stature, cultural norms and climate). Establishment of a locally-appropriate normal reference range allows for an accurate estimation of the impact of active TB on exercise tolerance.

St George’s Respiratory Questionnaire (SGRQ) has rarely been tested in ‘normal’ healthy populations, and some points in the questionnaire (such as one relating to impacts on quality of life of taking medications, see Appendix 15.6) are irrelevant in healthy volunteers. Nevertheless, evaluating the SGRQ in the local Timika population was necessary in order to test the performance of the modified, translated SGRQ, and to control for any potential locally-relevant factors which could influence results, such as cigarette smoking rates, lung damage due to pollution from indoor cooking fires, or culturally-determined perceptions of disability and quality of life. Most healthy volunteers scored zero, thus clearly indicating the high specificity of this questionnaire for quality of life impairment due to respiratory-induced disability, as well as allowing for an accurate baseline against which to estimate the impact of TB on quality of life.
When completed, data from the AVDAPT trial will be able to demonstrate whether the low-cost interventions of adjunctive L-arginine and vitamin D singly or together can shorten time to sputum clearance and culture negativity, which would have potential for public health benefit with reduced infectivity. Further, any acceleration in improvement in weight, symptoms and disability could enable an earlier return to work or school, thus translating to economic benefits to individuals and society. Even if only small benefits are observed, combining this with other recent TB developments (e.g. evolving TB drug regimens incorporating moxifloxacin, improved rifamycin formulations or doses, or TMC207 [discussed in Chapter 3]), could achieve more significant incremental improvements and contribute to much-needed improvements in standard TB treatment regimens. If adjunctive L-arginine and vitamin D are associated with no improvements, the trial will nevertheless provide answers to important research questions. Comparing mycobacterial clearance in each study arm will contribute to the current debate on the relative importance of the L-arginine-NO and vitamin D-cathelicidin pathways in mycobacterial killing in humans in vivo. In the current blinded analyses, the impact of L-arginine administration on FeNO is unable to be assessed: if L-arginine is subsequently found to increase FeNO and is associated with improved outcomes, this will provide mechanistic evidence for the effect. It would also identify FeNO as a readily measurable lung tissue immunological correlate of treatment outcome, useful in future clinical and immunological studies in TB. If L-arginine therapy improves T cell CD3ζ expression and T cell function (described in Chapter 3), this would support a dual effect of L-arginine therapy and provide the first evidence that this mechanism of impaired T cell function can be overcome with simple interventions potentially applicable to many other disease states.

Due to close links between MSHR-NIHRD’s Timika Translational Research Facility and national policy makers in Indonesia, the study findings will be readily able to be translated into policy and practice. The AVDAPT study will be eligible for inclusion by the Cochrane Infectious Diseases Collaborative Review Group.

This thesis has described the development and implementation of the AVDAPT study, and provided analyses of baseline data from enrolled participants. It has illustrated the persisting high impact of TB in the 21st century, and provided directions for future progress in the improvement of TB management.
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References


References


15 Appendices

15.1 STUDY INVESTIGATORS

Arginine and Vitamin D Adjunctive therapies in Pulmonary TB (AVDPAT) Investigators:

- **Dr Sandjaja**, PhD, Senior Researcher, Food and Nutrition Research and Development Center, National Institute for Health Research & Development (NIHRD), Bogor, Indonesia: Indonesian PI
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- **A/Prof Graham Maguire**, MBBS, PhD, FRACP, Respiratory Physician and Honorary Research Fellow, MSHR
- **A/Prof Peter Morris**, MBBS, PhD, FRACP, Clinical Trials Specialist and Research Fellow, MSHR
- **Dr Ivan Bastian**, MBBS, PhD, FRCPA, Microbiologist, WHO Supranational Reference laboratory, Institute for Medical & Veterinary Science (IMVS), Adelaide, Australia
- **Dr Michael Stone**, MD, Medical Advisor, Public Health Malaria Control, Timika, Papua, Indonesia.
- **Dr Ric Price**, MBChB, FRCP, PhD, Infectious Diseases Specialist, MSHR

Consultants:

- **Dr Jane Soepardi**, MD, Director, National Tuberculosis Control Program, Ministry of Health, Jakarta (all aspects of TB Program delivery)
- **Mr Richard Lumb**, BSc, TB Laboratory Scientist, WHO Supranational Reference laboratory, IMVS,IMVS, Adelaide (TB culture and sensitivity)
- **Prof Niels Becker**, BSc, MSc, PhD. Professor of Biostatistics, National Centre for Epidemiology & Population Health, ANU, Canberra, ACT, Australia. (statistical methods).
• **Dr Cheryl Salome**, PhD, Leader, Airway Physiology Group, Woolcock Institute of Medical Research, University of Sydney (exhaled NO measurements and quality control)
• **Prof John Eisman**, BSc (Med), MBBS, FRACP, PhD. Director, Bone and Mineral Research Program, Garvan Institute of Medical Research, Sydney Australia (monitoring effects of Vitamin D)
• **Dr Tonia Woodberry**, BSci, PhD, Laboratory scientist, MSHR (PBMC)
• **Dr Indri Rooslamiati**, BPharm, Laboratory scientist, NIHRD (PK/PD)
• **Prof Stephen Duffull**, MPharm, PhD, Professor of Pharmacy, University of Otago, Dunedin, New Zealand (PK/PD)
• **Dr Tsin Yeo**, MD, PhD Candidate, NIHRD-MSHR Research Collaboration (PK/PD)

**Data and Safety Monitoring Committee:**

• **Dr Paulus Sugiarto** MD, Chair of Safety monitoring committee. Clinical Director, RSMM, Timika
• **Dwi Hapsari Tjandrarini**, Biostatistician, NIHRD, Jakarta.
• **Dr Louise Maple-Brown**, MBBS FRACP PhD.: Senior Lecturer and Specialist, Internal Medicine and Endocrinology, MSHR
• **Dr Joseph McDonnell**, Statistician, Menzies School of Health Research.
COMPLETION CERTIFICATE

awarded to

Anna Ralph

in recognition of successful completion of

online Good Clinical Practice (GCP) course

Course AA. Site Personnel – Core GCP Training

Date of Completion 14/04/2008

This certificate is valid for 2 years from Date of Completion

ClinfoSource
Online Training for Clinical Trials

Nucleus network
Education
L-arginine and Vitamin D Adjunctive Therapy in Pulmonary Tuberculosis (TB)
(AVDAPT)

This study is currently recruiting participants.
Verified by Menzies School of Health Research, February 2010

<table>
<thead>
<tr>
<th>Sponsor:</th>
<th>Menzies School of Health Research</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collaborators:</td>
<td>National Institute for Health Research and Development, Indonesia</td>
</tr>
<tr>
<td></td>
<td>Australian National University</td>
</tr>
<tr>
<td>Information provided by:</td>
<td>Menzies School of Health Research</td>
</tr>
<tr>
<td>ClinicalTrials.gov Identifier:</td>
<td>NCT00677339</td>
</tr>
</tbody>
</table>

**Purpose**

The purpose of this study is to determine whether adjunctive L-arginine and vitamin D can improve response to standard short course TB therapy in people with newly diagnosed pulmonary TB.

<table>
<thead>
<tr>
<th>Condition</th>
<th>Intervention</th>
<th>Phase</th>
</tr>
</thead>
</table>
| Smear Positive Pulmonary Tuberculosis | Drug: L-arginine  
Drug: Vitamin D  
Drug: Placebo L-arginine  
Drug: Placebo Vitamin D | Phase 3   |

Study Type: Intervventional
Study Design: Treatment, Factorial Assignment, Double Blind (Subject, Caregiver, Investigator, Outcomes Assessor), Randomized, Placebo Control, Safety/Efficacy Study
Official Title: Phase 3 Trial of Oral L-arginine and / or Vitamin D as Adjunctive Therapies in Pulmonary Tuberculosis in Papua Province, Indonesia.
Further study details as provided by Menzies School of Health Research:

Primary Outcome Measure:

- Proportion of pulmonary TB patients who are culture negative at 1 month  [Time Frame: 1 month]  [Designated as safety issue: No]
- Difference in improvement in composite clinical endpoint comprising weight, cough clearance and FEV1 at 2 months.  [Time Frame: 2 months]  [Designated as safety issue: No]

Secondary Outcome Measures:

- Change in plasma L-arginine concentration  [Time Frame: week 0, 2, 4, 8, 24]  [Designated as safety issue: Yes]
- Change in plasma 25(OH)D3 concentration  [Time Frame: week 0, 2, 4, 8, 24]  [Designated as safety issue: Yes]
- Death, clinical failure and default independently, and 'death or clinical failure or default'.  [Time Frame: week 24]  [Designated as safety issue: Yes]
- Hypercalcaemia  [Time Frame: week 0, 2, 4, 8, 24]  [Designated as safety issue: Yes]
- Gastrointestinal side effects  [Time Frame: weekly to week 8 then at week 24]  [Designated as safety issue: Yes]
- Sputum smear conversion time  [Time Frame: weekly to week 8 then at week 24]  [Designated as safety issue: No]
- Radiological improvement (percentage lung involvement on CXR at 2 months).  [Time Frame: week 0, 2, 4, 8, 24]  [Designated as safety issue: No]
- Cough clearance  [Time Frame: weekly to week 8 then at week 24]  [Designated as safety issue: No]
- Difference in improvement in percent predicted FEV1 at 2 and 6 months.  [Time Frame: weeks 0, 4, 8, 24]  [Designated as safety issue: No]
- Weight gain  [Time Frame: weekly to week 8 then at week 24]  [Designated as safety issue: No]
- Immunological improvement (exhaled NO)  [Time Frame: week 0, 2, 4, 8, 24]  [Designated as safety issue: No]
- Immunological improvement (T cell CD3ζ expression and T cell function)  [Time Frame: week 0, 2, 4, 24]  [Designated as safety issue: No]
- Functional improvement measured using six minute walk test  [Time Frame: week 0, 4, 8, 24]  [Designated as safety issue: No]
- Quality of life assessment using modified St George Respiratory Questionnaire.  [Time Frame: weeks 0, 4, 8, 24]  [Designated as safety issue: No]
- Primary end points stratified by HIV status.  [Time Frame: weekly to week 8 then at week 24]  [Designated as safety issue: No]
- Primary end points stratified by baseline vitamin D and L-arginine status.  [Time Frame: weekly to week 8 then week 24]  [Designated as safety issue: No]
- Primary end points stratified by ethnicity (Papuan and non-Papuan patients).  [Time Frame: weekly to week 8 then week 24]  [Designated as safety issue: No]

Estimated Enrollment: 444
Study Start Date: June 2008
Estimated Study Completion Date: June 2012
Estimated Primary Completion Date: December 2011
<table>
<thead>
<tr>
<th>Arms</th>
<th>Assigned Interventions</th>
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<tbody>
<tr>
<td>Active Comparator: 1</td>
<td>Drug: L-arginine</td>
</tr>
<tr>
<td>Active L-arginine plus active vitamin D</td>
<td>L-arginine 6g orally daily</td>
</tr>
<tr>
<td></td>
<td>Drug: Vitamin D</td>
</tr>
<tr>
<td></td>
<td>Cholecalciferol 50000 IU once monthly orally</td>
</tr>
<tr>
<td>Active Comparator: 2</td>
<td>Drug: Vitamin D</td>
</tr>
<tr>
<td>Placebo L-arginine plus active Vitamin D</td>
<td>Cholecalciferol 50000 IU once monthly orally</td>
</tr>
<tr>
<td></td>
<td>Drug: Placebo L-arginine</td>
</tr>
<tr>
<td></td>
<td>placebo L-arginine once daily</td>
</tr>
<tr>
<td>Active Comparator: 3</td>
<td>Drug: L-arginine</td>
</tr>
<tr>
<td>Active L-arginine plus placebo vitamin D</td>
<td>L-arginine 6g orally daily</td>
</tr>
<tr>
<td></td>
<td>Drug: Placebo Vitamin D</td>
</tr>
<tr>
<td></td>
<td>placebo vitamin D orally once monthly</td>
</tr>
<tr>
<td>Placebo Comparator: 4</td>
<td>Drug: Placebo L-arginine</td>
</tr>
<tr>
<td>placebo L-arginine plus placebo vitamin D</td>
<td>placebo L-arginine once daily</td>
</tr>
<tr>
<td></td>
<td>Drug: Placebo Vitamin D</td>
</tr>
<tr>
<td></td>
<td>placebo vitamin D orally once monthly</td>
</tr>
</tbody>
</table>

The two major pathways proposed to mediate macrophage mycobacterial killing in humans are the arginine-nitric oxide and Vitamin D-1,25 dihydroxyvitamin D pathways. Our aim is to determine if the key immunomodulatory agents L-arginine and vitamin D can improve the rapidity and magnitude of the microbiological and clinical response in pulmonary TB. We will test the following hypotheses in newly-diagnosed TB patients in Timika, Papua, Indonesia:

Our specific aims are to:

1. Determine whether supplementation with L-arginine and/or vitamin D is safe, and results in more rapid improvement in clinical, mycobacterial, immunological, radiological, physiological and functional measures of treatment outcome. We will randomise patients with pulmonary TB to receive, in addition to standard TB therapy, adjunctive arginine, vitamin D and / or placebo in a randomised, double-blind factorial 2x2 design. We will relate serial measurements of plasma concentrations of L-arginine and vitamin D, and immunological responses (pulmonary NO production, T cell function and phenotype) to measures of treatment outcome [mycobacterial (sputum smear clearance and culture conversion), physiological (spirometry), clinical (symptoms and weight), radiological (chest Xray) and functional (six-minute walk test, modified St George Respiratory Questionnaire)].

2. Determine whether pulmonary production of NO is inversely related to disease severity at presentation. Baseline and serial measures of NO production will be related to disease severity and the magnitude and rapidity of clinical response
Eligibility

Ages Eligible for Study: 15 Years and older
Genders Eligible for Study: Both

Inclusion Criteria:

• Adults >15 years with sputum smear positive pulmonary TB
• New cases only
• Agree to continue treatment in Timika for the full six month course of treatment - Not pregnant
• Consent to enroll in the study.

Exclusion Criteria:

• hypercalcaemia (ionized calcium >1.32 mmol/L) identified at baseline
• taking arginine or vitamin D

Contacts and Locations

Contacts
Anna P Ralph, MBBS 61-2-6125-0538 anna.ralph@anu.edu.au
Nicholas M Anstey, MBBS 61-8-8922-8942 anstey@menzies.edu.au

Locations
Indonesia, Papua Province
Timika Tuberculosis Clinic and Community Hospital Recruiting
Timika, Papua Province, Indonesia
Contact: Govert Waramori 0011 62 811 495 418 Govert_Waramori@fmi.com
Sub-Investigator: Govert Waramori
Sub-Investigator: Enny Malonda, MD
Sub-Investigator: Andri Wiguna, MD
Sub-Investigator: Pasi Penttinen, MD

Investigators
Study Director: Sandjaja, PhD National Institute for Health Research and Development, Indonesia

Study Director: Nicholas M Anstey, MBBS Menzies School of Health Research

Study Director: Paul M Kelly, MBBS Australian National University, Canberra, Australia

Principal Investigator: Dina B Lalong, MD National Institute of Health Research & Development, Indonesia

Principal Investigator: Anna P Ralph, MBBS Australian National University,
Principal Investigator: Emiliana Tjitra, MD
Canberra, Australia
National Institute for Health Research & Development, Indonesia

Principal Investigator: Franciscus Thio, MPPM
District Ministry of Health, Timika

Principal Investigator: Peter Morris, MBBS
Menzies School of Health Research, Northern Territory, Australia

Principal Investigator: Graeme Maguire, MBBS
Menzies School of Health Research, Northern Territory, Australia

More Information

Responsible Party: Menzies School of Health Research, NT (Nicholas Anstey, Head of International Division)

Study ID Numbers: AVDAPT 1

Health Authority: Indonesia: National Agency of Drug and Food Control
15.4 AVDAPT INFORMATION AND CONSENT FORMS

AVDAPT Study Participant / Guardian information sheet

Arginine and Vitamin D Adjuvant Therapies in Pulmonary Tuberculosis (AVDAPT)
Research study at Timika Puskesmas and RS Mitra Masyarakat

Study Participant / Guardian information sheet

Can extra medicine help make TB better more quickly?

This is for you to keep

Tuberculosis (TB) is a major cause of sickness in Indonesia. It can cause cough, weight loss and other problems. People with TB need to take antibiotics for a long time, usually 6 months, to be cured. We want to find out if new extra treatments taken with the antibiotics might help patients with TB to get better faster. We are asking people with lung TB to take part in this study. If you agree, we will ask you to take extra tablets for 2 months in addition to the usual TB antibiotics (which you take for 6 months). We will also ask you to do some extra lung and blood tests today, and again in 2, 4, 8 and 24 weeks.

The body needs nutrition, such as protein and vitamins, to stay healthy and help fight off infection. There is a substance all people need called arginine which is found in foods like nuts, but also comes in a tablet. Also, an important vitamin is Vitamin D which comes from sunshine through the skin, and also from some foods, and can be taken as a tablet too. Some doctors think that extra arginine and vitamin D may be good for people with TB, but there is no clear answer to this yet.

You can help us try to answer this important question. If you agree to be part of this study, you will be given some extra tablets. Some people will receive tablets that have the real arginine or vitamin D, and some people will be given tablets that contain nothing except powder (‘placebo’ tablet). You, your doctor and the study investigators are not allowed to know until the end which you are taking, to make sure the results are not biased. If you are taking the real arginine and / or vitamin D, they should not cause you any harm. It is very uncommon, but some people get stomach upset from arginine, so we will be checking for this and advise to take tablets after food. Vitamin D can rarely make the calcium in blood high, so we will be checking for this in your blood tests. If you have high calcium already, or get high calcium later, we will stop the extra medications.

Whether or not you agree to participate in this study, you will receive the standard TB antibiotics that the health centre usually uses to treat people with tuberculosis. As part of normal care, the TB clinic will ask you to provide a sputum sample, have your height and weight measured, get a chest x ray and have blood taken (including an HIV test) and you will be asked questions about your health.

If you say yes to the study, then when the blood is taken, we will collect an additional 20 ml of blood (4 teaspoons). This will include testing for HIV if you agree, which is standard care for all people with TB. Then we will also ask you to do breathing tests, a walking test for 6 minutes, and answer some more questions about your breathing. When your blood test results are ready, you will get the extra medications. For the lung tests, we will ask you to breathe into the mouthpiece of a machine called a spirometer and a NIOX machine. This does not hurt or cause any harm, but tells us how your lungs are working. As part of routine care by the TB clinic,
every week you will have a check up to see how you are feeling and check your weight. If you are in the study, you will also be asked to do the following tests: sputum test every week; NIOX lung test and blood test in 2, 4, 8 and 24 weeks; chest x ray, spirometer lung test, walk test and questionnaire in 4, 8 and 24 weeks.

Taking part in this study is voluntary; it does not cost you any money. All information collected is confidential. Results will be given to your doctor if they can help the doctor to treat your illness. No personal identification will be revealed to persons outside the study. You do not have to participate if you don’t want to, this will not affect your medical treatment. If you decide to participate, you can withdraw from this study at any time for any reason and still receive standard treatment for tuberculosis by the staff at the health centre.

If you have any questions about the study you can telephone Dr Paulus Sugiarto at RS Mitra Masyarakat Hospital on 901-301 881. If you have any concerns or complaints about the conduct of the study, please contact Dr Sandjaja, Chair of the NIHRD Ethics Committee in Jakarta on 081319304040 or 6202104261088, or The Secretary, Human Research Ethics Committee of the NT Department of Health & Community services & Menzies School of Health Research in Australia on 61-8-8922 7922.
Can extra medicine help make TB better more quickly?

This form means you can say No.

I have read the Patient Information Sheet and have had the details explained to me by the witness below. I understand that I will receive the usual treatment and tests for tuberculosis. I understand that I will also have extra tablets which may be active arginine and / or vitamin D, or placebo. I will also have extra lung tests, blood tests, sputum tests, repeat chest X-rays and questions that are not needed by my doctors to treat my tuberculosis. The extra tablets are being tested to find out if they will help people with TB to get better faster. They are very unlikely to be harmful, but could potentially cause stomach upset or high calcium level. The extra tests will provide information about how my lungs and blood are working. I may not receive any direct benefit from this study, but the results will help answer questions about TB treatment, and therefore may be of help to other people in the future.

I understand that I have tuberculosis and I will be asked to come to Timika TB Clinic / RSMM (delete one) for repeat breathing tests, blood tests, chest X-ray, walk test and questionnaire in 2, 4, 8 and 24 weeks. I understand that this will take longer than a usual clinic appointment. I also understand that my medical records at the health centre will be read by research staff and some information about my health will be collected.

I understand that all information collected is confidential and no information will be available to anyone outside the study. Results will be given to my doctor if they can help the doctor to treat my illness. I understand that I do not have to participate in this study. I can withdraw from this study at any time for any reason and still receive standard treatment for tuberculosis by the staff at Rumah Sakit Mitra Masyarakat or Timika TB Clinic.
PARTICIPANT
I (print name) ................................................................. agree to take part in this study.
Signed ........................................................................... Date
......................................................................................... _____ / _____ / 20_____ 

PARTICIPANTS AGED <18 YEARS: PARENT / GUARDIAN TO SIGN
I (print name) ................................................................. agree for my child / person in my care to take part in this study.

Participant name ..................................................................
Signed ........................................................................... Date
......................................................................................... _____ / _____ / 20_____ 

WITNESS
I (print name) ................................................................. have explained the study & information sheet
Signed ........................................................................... Date
......................................................................................... _____ / _____ / 20_____ 

Page 2 of 2
**Bagian A: Kriteria Inklusi dan Eksklusi**  
Section A: Inclusion and Exclusion Criteria

<p>| | | | | | | |</p>
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</thead>
<tbody>
<tr>
<td>Nama peserta [pertama]:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td>First</td>
<td>Surname</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanggal hari ini:</td>
<td></td>
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<td></td>
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<tr>
<td>Date today</td>
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<tr>
<td>Nama pewawancara:</td>
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<tr>
<td>Data collector</td>
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</tr>
</tbody>
</table>

1. **BTA positif TB paru:** Smear positive pulmonary TB  
   - Ya [ ]  
   - Tidak [ ]

2. **TB Baru:** Never treated for TB before  
   - Ya [ ]  
   - Tidak [ ]

3. **Bersedia untuk menjalani pengobatan di Timika selama 6 bulan:** Agree to continue treatment in Timika for 6 months  
   - Ya [ ]  
   - Tidak [ ]

4. **Umur >= 15 th:** Age > OR = 15yrs  
   - Ya [ ]  
   - Tidak [ ]

5. **Persetujuan Consent untuk berpartisipasi dalam penelitian ini:** Consent given  
   - Ya [ ]  
   - Tidak [ ]

   **Bila menjawab “tidak” untuk pertanyaan no. 1-5, pasien tidak bisa dimasukkan dalam penelitian ini.**  
   *If ‘No’ to any questions 1 to 5, PATIENT IS NOT ELIGIBLE*

6. **Apakah dalam keadaan hamil?** Pregnant?  
   - Ya [ ]  
   - Tidak [ ]

7. **Ionized calcium > 1.32 mmol/L?**  
   - Ya [ ]  
   - Tidak [ ]

   **Bila pasien menjawab “Ya” untuk pertanyaan no. 6-7, pasien tidak bisa diikutkan dalam penelitian ini.**  
   *If ‘Yes’ to question 6-7, PATIENT IS NOT ELIGIBLE*
**Bagian B: Informasi dasar peserta**

Section B: Respondent Baseline Information

**Tempat:**
- TB Clinic [1]
- RSMM [2]
- Puskesmas [3]
- Other [4]

**Tanggal lahir:** [___] / [___] / 19[___]

**Umur:** [___] thn

**Alamat:** ___________________

**Telepon:** ___________________

**Jenis Kelamin:**
- Laki [1]
- Perempuan [0]

**Suku:**
- Papuan [1]
- Non-Papuan [0]

**Ethnicity**
- Agats [ ]
- Amungme [1]
- Asmat [ ]
- Ayamaru [2]
- Biak [ ]
- Bintuni [3]
- Damal [ ]
- Dani [4]
- Ekari [9]
- Fak-Fak [10]
- Jayapura [12]
- Kamoro [13]
- Dani [15]
- Mee [16]
- Merauke [17]
- Moni [18]
- Nabire [19]
- Nduga [20]
- NonPapuan [21]
- Paniai [22]
- Sarui [23]
- Sorong [24]
- Ekari [9]
- Merauke [17]
- Lain [25]

**Pendidikan:**
- Sekolah Dasar [1]
- SMP/SMA [2]
- Akademi/Universitas [3]
- Tidak sekolah [4]

**Pekerjaan:**
- Bekerja [1]
- Pergi ke sekolah [2]
- Tidak ada [3]

**Riwayat merokok?**
- Merokok [1]
- Pernah merokok tetapi sudah berhenti [2]
- Tidak pernah merokok [3]

Investigator name and signature: ________________________________
Jika merokok, cigs/day? ______

Berapa banyak rokok yang diisap per hari? ______

Sudah berapa lama sakit seperti ini? How long have you have this sickness? ______ bulan

Tinggi badan Height ______ m

FORMULIR PENGUMPULAN DATA MINGGUAN Weekly data collection form

Bagian A: Data Peserta

Nama peserta [pertama]: [keluarga]: ____________________

Name First Surname

Tanggal: ____ | ____ | 20______ Minggu pengobatan (0-24) ____ | ____

Date today Week of treatment

Nama pengumpul data: _________________________________

Data collector

Berapa bungkus obat yang dihabiskan selama minggu ini? How many tablet packs taken?

Obat TB program 1 Arginine / placebo 1

Second Vitamin D / placebo dose given today? Ya Tidak

If given on other day during this week, date given:

_____ | ____ | 20______

Comments regarding mediations: ________________________________________________

Seberapa sering pergi ke clinic? Frequency of clinic visit

Setiap hari 1 every day

Setiap minggu 2 every week

Setiap bulan 3 every month

Lain-lain 4 other:

Bagian B : Petunjuk Langkah Demi Langkah Pengkajian Mingguan

Jam berapa makan terakhir? What time did patient eat?

Waktu pengambilan darah? What time was blood taken

(dicatat juga di tabung) (dicatat juga di tabung)

Waktu pemeriksaan eNO What time eNO measured?

Catat hasil pemeriksaan darah iSTAT

Ionised calcium ______ mmol/L

Haemoglobin ______ g/dL

Investigator name and signature: ________________________________
## Bagian C: Gejala
**Section C: Symptoms**

### Berat badan
**Weight**

<table>
<thead>
<tr>
<th>Kg</th>
<th>Ya</th>
<th>Tidak</th>
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</thead>
</table>

### Batuk
**Cough**

- Jika ada batuk:
  - Ringan
  - Sedang
  - Parah
  - Parah sekali

<table>
<thead>
<tr>
<th>1 Mild</th>
<th>2 Mod</th>
<th>3 Severe</th>
<th>4 Very severe</th>
</tr>
</thead>
</table>

- Apakah ada perubahan?
  - Memburu
  - Sama
  - Lebih baik
  - Hilang

<table>
<thead>
<tr>
<th>1 Worse</th>
<th>2 Same</th>
<th>3 Better</th>
<th>4 Gone</th>
</tr>
</thead>
</table>

- Apakah ada lendir?
  - Ya | Tidak |

- Apakah batuk darah?
  - Ya | Tidak |

### Demam
**Fever**

<table>
<thead>
<tr>
<th>Ya</th>
<th>Tidak</th>
</tr>
</thead>
</table>

### Lemas
**Malaise**

<table>
<thead>
<tr>
<th>Ya</th>
<th>Tidak</th>
</tr>
</thead>
</table>

### Bingung
**Confusion**

<table>
<thead>
<tr>
<th>Ya</th>
<th>Tidak</th>
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### Kembung
**Bloating**

<table>
<thead>
<tr>
<th>Ya</th>
<th>Tidak</th>
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### Konstipasi
**Constipation**

<table>
<thead>
<tr>
<th>Ya</th>
<th>Tidak</th>
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### Mual
**Nausea**

<table>
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<tr>
<th>Ya</th>
<th>Tidak</th>
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### Muntah
**Vomiting**

<table>
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<tr>
<th>Ya</th>
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### Diare
**Diarrhoea**

<table>
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<th>Ya</th>
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### Sakit kepala
**Headache**

<table>
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<tr>
<th>Ya</th>
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### Pusing
**Dizziness**

<table>
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<tr>
<th>Ya</th>
<th>Tidak</th>
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</table>

### Jaundice
**Kuning**

<table>
<thead>
<tr>
<th>Ya</th>
<th>Tidak</th>
</tr>
</thead>
</table>

---

Investigator name and signature: ________________________________

198
AVDAPT

Kemerahan
Rash
Ya ☐  Tidak ☐

Gatal-gatal
Itch
Ya ☐  Tidak ☐

Rasa terbakar, mati rasa di tangan & kaki
Burning, numbness in feet or hands
Ya ☐  Tidak ☐

Gangguan penglihatan
Poor vision, colour blind
Ya ☐  Tidak ☐

Gangguan persendian
Joint pain/ swelling
Ya ☐  Tidak ☐

Berat badan turun
Weight loss
Ya ☐  Tidak ☐

Mialgia
Myalgia
Ya ☐  Tidak ☐

Lain-lain, sebutkan
Ya ☐  Tidak ☐

Other, specify

Lain-lain, sebutkan
Ya ☐  Tidak ☐

Other, specify

Lain-lain, sebutkan
Ya ☐  Tidak ☐

Other, specify

HIV status known
Ya ☐  Tidak ☐

If known: Result date  ____ | ____ | 20____
If known: CD4 count  |____|____|

Apakah anda minum obat untuk lain-lain?
Ya ☐  Tidak ☐

Are you taking any other medications?

Jika ja, obat apa?

What medicines?

<table>
<thead>
<tr>
<th></th>
<th>Ya ☐</th>
<th>Tidak ☐</th>
<th>Tanggal:  ____</th>
<th>____</th>
<th>20____</th>
</tr>
</thead>
<tbody>
<tr>
<td>D4T</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>3TC</td>
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<tr>
<td>AZT</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nevirapine</td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Efavirenz</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cotrimoxazol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Investigator name and signature: _____________________________________________

199
Obat lain

Investigator name and signature: _____________________________________________

200
Nama peserta [pertama]: __________________________ [keluarga]: __________________________

Tanggal:   |__|__|/|__|__|/20__|__|   Minggu |____|

Nama pewawancara: __________________________

**Meal**

- Time of last meal   |__|__| : |__|__|

**Time 0: Baseline**

- eNO time   |__|__| : |__|__|   eNO result
  |__|__| | ppB
- blood time   |__|__| : |__|__|

**Time 1: Medications given**

- tablet time   |__|__| : |__|__|

**Time 2: Follow-up reading**

- eNO time   |__|__| : |__|__|   eNO result
  |__|__| | ppB
- blood time   |__|__| : |__|__|

**Time 3: Follow-up reading**

- eNO time   |__|__| : |__|__|   eNO result
  |__|__| | ppB
- blood time   |__|__| : |__|__|

Investigator name and signature: ____________________________
<table>
<thead>
<tr>
<th>X-ray date</th>
<th>Total % affected lung</th>
<th>Cavitation</th>
<th>Effusion</th>
<th>Any consolidation</th>
<th>Any nodules</th>
<th>Any fibrosis</th>
<th>Miliary disease</th>
<th>Score</th>
<th>Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>___</td>
<td>___</td>
<td>20____</td>
<td>___</td>
<td>___</td>
<td>20____</td>
<td>___</td>
<td>___</td>
<td>20____</td>
<td>___</td>
</tr>
<tr>
<td>%</td>
<td>%</td>
<td>%</td>
<td>Absent</td>
<td>Mod (≤4cm)</td>
<td>Severe (&gt;4cm)</td>
<td>Absent</td>
<td>Mod (≤4cm)</td>
<td>Severe (&gt;4cm)</td>
<td>Absent</td>
</tr>
<tr>
<td>Absent</td>
<td>Small (&lt;25% of lung field)</td>
<td>Large (≥25% of lung field)</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
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<td>Yes</td>
<td>No</td>
<td>No</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
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<th>___________________________________________________________________</th>
<th>___________________________________________________________________</th>
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</table>

<table>
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<tr>
<th>Reporter name and signature</th>
<th>___________________________________________________________________</th>
<th>___________________________________________________________________</th>
<th>__________________</th>
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<table>
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<tr>
<th>Date of report</th>
<th>___</th>
<th>___</th>
<th>20____</th>
<th>___</th>
<th>___</th>
<th>20____</th>
<th>___</th>
<th>___</th>
<th>20____</th>
</tr>
</thead>
</table>

Investigator name and signature: ________________________________________________
Nama peserta [pertama]: ____________________ [keluarga]: ____________________
Name ____________________ Surname ____________________

Tanggal:  |_ | |_ | / |_ | |_ | / 20__ |
Date ____________________

Outcome: ____________________
(tick one) ____________________
Pengobatan selesai  
Completed  
1  
Sembuh  
Cured  
2  
Gagal pengobatan  
Failed (smear positive at 5 months)  
3  
Default  
Defaulted  
4  
Meninggal  
Died  
5  
Transfer  
Tranferred  
6

Investigator name and signature: ____________________________________________
**PENGKAJIAN TERAKHIR**

**Outcome**

| ENROLMENT DATE: |   |   | 20 |   |
| DATE LAST SEEN: |   |   | 20 |   |

**Bacteriology**

<table>
<thead>
<tr>
<th>Week</th>
<th>UI Smear</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>8</td>
<td>0</td>
<td>3</td>
</tr>
</tbody>
</table>

**Outcome**

- Cured (2 consec. smear -) [ ] 1 [ ]
- Completed [ ]
- Failed (smear + 5 mo) [ ] 2 [ ]
- Defaulted [ ] 3 [ ]
- Died [ ] 4 [ ]
- Transferred [ ] 5 [ ]

**TB Treatment Regimen**

- Non-standard: Non FDC [ ] 1 [ ] Yes [ ] No [ ] 0
- Non-standard: >24 weeks [ ] 2 [ ] Yes [ ] No [ ] 0
- Non-standard: Other [ ] 3 [ ] Yes [ ] No [ ] 0

**Protocol Violations**

- Enrolment violation [ ] 1 [ ] Yes [ ] No [ ] 0
- Randomisation violation [ ] 2 [ ] Yes [ ] No [ ] 0
- Study drug error (mislabelled, missing) [ ] 3 [ ] Yes [ ] No [ ] 0
- Follow up missed: 1-2 appointments [ ] 4 [ ] Yes [ ] No [ ] 0
- Follow up missed: >=3 appointments [ ] 5 [ ] Yes [ ] No [ ] 0
- Compliance <80% [ ] 6 [ ] Yes [ ] No [ ] 0
- Study drugs stopped by investigator [ ] 7 [ ] Yes [ ] No [ ] 0
- Consent withdrawn before week 8 [ ] 8 [ ] Yes [ ] No [ ] 0
- Consent withdrawn after week 8 [ ] 9 [ ] Yes [ ] No [ ] 0
- Second VITD/placebo dose administration [ ] 10 [ ] At week 4 [ ] 1 [ ] Yes [ ] No [ ] 0
- After week 4 [ ] 2 [ ] Yes [ ] No [ ] 0
- Not given [ ] 3 [ ] Yes [ ] No [ ] 0
- Not recorded [ ] 4 [ ] Yes [ ] No [ ] 0

**Other Details:**

- Other details: [ ] [ ] Yes [ ] No [ ] 0

**Adverse Events**

**Hypercalcaemia**

- No [ ] 0 [ ] 0
- Mild 1.33-1.39 [ ] 1 [ ] 1
- Moderate 1.40-1.49 [ ] 2 [ ] 2
- Severe >1.50 [ ] 3 [ ] 3

**Other Adverse Event**

- Serious adverse event [ ] 1 [ ] Yes [ ] No [ ] 0

**HIV**

- Negative [ ] 0 [ ] 0
- Positive [ ] 1 [ ] 1
- Indeterminate [ ] 2 [ ] 2
- Not done [ ] 3 [ ] 3

Investigator name and signature: ____________________________________________
15.6 ST GEORGE’S RESPIRATORY QUESTIONNAIRE

English translation

SGRQ PART 1

1) Over the last year, I have coughed:
Most days
Several
A few
Only ______ days
Not at all

2) Over the last year, I have brought up phlegm (sputum):
Most days
Several
A few
Only ______ days
Not at all

3) Over the last year, I have had shortness of breath:
Most days
Several
A few
Only ______ days
Not at all

4) Over the last year, I have had attacks of wheezing:
Most days
Several
A few
Only ______ days
Not at all

5) During the last year, how many severe or very bad unpleasant attacks of chest trouble have you had?
More than three
3 attacks
2 attacks
1 attack
None

6) How long did the worst attack of chest trouble last?
a week or more
3 or more days
1 or 2 days
less than a day

7) Over the last year, in an average week, how many good days (with little chest trouble) have you had?
None
1 or 2
3 or 4
nearly every day
every day

8) If you have a wheeze, is it worse in the morning?
No
Yes

SGRQ PART 2

9) How would you describe your chest condition?
The most important problem I have
Causes me quite a lot of problems
Causes me a few problems
Causes no problem

10) If you have ever had paid employment?
My chest trouble made me stop work
My chest trouble interferes with my work or made me change my work
My chest trouble does not affect my work

11) Questions about what activities usually make you feel breathless.
Sitting or lying still
Getting washed or dressed
Walking around the home
Walking outside on the level
Walking up a flight of stairs
Walking up hills
Playing sports or games
12) More questions about your cough and breathlessness.
My cough hurts
My cough makes me tired
I get breathless when I talk
I get breathless when I bend over
My cough or breathing disturbs my sleep
I get exhausted easily

13) Questions about other effects your chest trouble may have on you.
My cough or breathing is embarrassing in public
My chest trouble is a nuisance to my family, friends or neighbours
I get afraid or panic when I cannot get my breath
I feel that I am not in control of my chest problem
I do not expect my chest to get any better
I have become frail or an invalid because of my chest
Exercise is not safe for me
Everything seems too much of an effort

14) Questions about your medication.
My medication does not help me very much
I get embarrassed using my medication in public
I have unpleasant side effects from my medication
My medication interferes with my life a lot

15) Questions about how activities may be affected by your breathing.
I take a long time to get washed or dressed
I cannot take a bath or shower, or I take a long time
I walk more slowly than other people, or I stop for rests
Jobs such as housework take a long time, or I have to stop for rests
If I walk up one flight of stairs, I have to go slowly or stop
If I hurry or walk fast, I have to stop or slow down
My breathing makes it difficult to do things such as walk up hills, carry things up stairs, light gardening such as weeding, dance, play bowls or play golf
My breathing makes it difficult to do things such as carry heavy loads, dig the garden or shovel snow, jog or walk at 5 miles per hour, play tennis or swim
My breathing makes it difficult to do things such as very heavy manual work, run, cycle, swim fast or play competitive sports

16) We would like to know how your chest trouble usually affects your daily life.
I cannot play sports or games
I cannot go out for entertainment or recreation
I cannot go out of the house to do the shopping
I cannot do housework
I cannot move far from my bed or chair

17) Tick the statement which you think best describes how your chest affects you.
It does not stop me doing anything I would like to do
0 It stops me doing one or two things I would like to do
It stops me doing most of the things I would like to do
It stops me doing everything I would like to do
PRODUCT DATA SHEET

15.7 ARGIMAX CERTIFICATE OF ANALYSIS

General information

Product name Argimax
Dosage form tablet
Product number 5146
Manufacturer Hankintatukku Oy, Finland
Package 60 tablets / 3 x 20 blister packed tablets in a carton

Ingredients

<table>
<thead>
<tr>
<th>Raw material</th>
<th>mg/tablet</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-argininehydrochloride (food quality)</td>
<td>1000,00</td>
<td>86,21</td>
</tr>
<tr>
<td>Microcrystalline cellulose (Ph. Eur.)</td>
<td>100,00</td>
<td>8,62</td>
</tr>
<tr>
<td>Hydroxypropyl cellulose (food quality)</td>
<td>48,00</td>
<td>4,14</td>
</tr>
<tr>
<td>Magnesium stearate (E 470b, Ph. Eur.)</td>
<td>12,00</td>
<td>1,03</td>
</tr>
<tr>
<td></td>
<td>1 160 mg</td>
<td>100 %</td>
</tr>
</tbody>
</table>

**label claim / 1 tablet contains**
830 mg L-arginine

Specification of the finished product

<table>
<thead>
<tr>
<th>Test</th>
<th>Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td>Appearance</td>
<td>White, 20 mm long, 8 mm broad, capsule formed tablet</td>
</tr>
<tr>
<td>Weight</td>
<td>1 160 mg (Ph. Eur.)</td>
</tr>
<tr>
<td>Checking of the carton / label</td>
<td>The right batch number and best before -marking</td>
</tr>
<tr>
<td>Content of the package</td>
<td>60 capsules in a carton</td>
</tr>
</tbody>
</table>
13th May 2008

RE: Cal D Forte Tablets Batch 90450

Please be advised that the batch number 90350 on the Processed Product Analytical Report is the manufacturing batch number of the tablets that were packed into Packing Batch Number 90450.

Kind Regards

Hugh Lazarus
Compliance Manager
PSM Healthcare Ltd trading as API Consumer Brands
**PRODUCT:** CALCIFEROL STRONG TABLETS – CORES  
**PRODUCT CODE:** CALC 125  
**CLIENT:** INTERNAL

<table>
<thead>
<tr>
<th>Description</th>
<th>Visual</th>
<th>8mm deep concave, brown tablet.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average Tablet Weight</td>
<td>Balance</td>
<td>195 (200) 205mg</td>
</tr>
</tbody>
</table>
| Weight Variation | BP Appendix XII G | Max wt: 208.9 mg (+3.4%)  
Min wt: 193.8 mg (-4.6%) |
| Identification | Brownish red colour produced | No tablet lies outside ± 15% of the average tablet weight |
| Hardness | BP ID B | 2 -- 7kp |
| Thickness | Dr Schlesinger | 4.81mm |
| Disintegration | BP Appendix XII A | Not more than 30 minutes |
| Loss on Drying | BP Appendix XVIIG | 100 - 105°C, 3h  
5. 000% |
| Friability | Cholecalciferol | Not more than 1.0%. No capping. |
| Calciferol | BP | 1.12 – 1.56 mg/tab  
1.20 mg/tab |

**ACTIVE INGREDIENT:** Cholecalciferol  
**RETIRED SAMPLE SIZE:** 100 Tablets

**BATCH APPROVED TO RELEASE BY:** Peter Taylor  
**DATE:** 14/1/08
15.9 SAFETY REPORTING PROCESS

**Clinical Investigator:** Determine if an Event is Serious (definitions on page 2)

---

**Yes**

- **Serious Event Reporting**
  - **Research Assistant in Timika:**
    - Determine causality
    - Notify Dr Anna Ralph and Dr Dina Bisara Lolong **WITHIN 24 HOURS**. If Anna not available, notify Dr Paul Kelly or Nick Anstey.
    - Record on SAE form

- **Dr Anna Ralph and Dr Dina Lolong:**
  - Ensure that event is recorded in safety database
  - Determine reportability. Is the event:
    - Serious AND
    - Unexpected AND
    - Related to study drug?

---

**No**

- **NonSerious Event Reporting**
  - **Research Assistant in Timika:**
    - Record on Weekly Data collection form
    - Discuss with TB clinic doctor and Study Investigators if medical advice needed
    - Notify BPOM in routine report
    - Record event in clinical database

---

**Yes**

- **Is the event: Fatal or life-threatening?**
  - **Yes**
    - Dr Anna Ralph and Dr Dina Lolong should notify and supply the SAE form **WITHIN 24 HOURS** to the Data Safety Monitoring Committee by fax or email, who will prepare a report for HREC and decide whether BPOM needs to be notified **WITHIN 7 DAYS**.
    - Other Investigators should be notified as soon as possible

  - **No**
    - **Dr Anna Ralph and Dr Dina Lolong** should notify and supply the SAE form **WITHIN 24 HOURS** to the Data Safety Monitoring Committee, who will prepare a report for HREC and decide whether BPOM needs to be notified **WITHIN 15 DAYS**
    - Other Investigators should also be notified within 15 calendar days

---

**No**

- **Dr Anna Ralph and Dr Dina Lolong** should notify and supply the SAE form **WITHIN 24 HOURS** to the Data Safety Monitoring Committee, who will prepare a report for HREC and decide whether BPOM needs to be notified **WITHIN 15 DAYS**

---

**Contacts**
- Dr Anna Ralph, fax + 61 2 6125 0740, email anna.ralph@anu.edu.au
- Dr Dina Bisara Lolong, fax 62 21 424 5386, email dina_lolong@litbang.depkes.go.id
- Dr Paul Kelly, fax + 61 2 6125 0740, email paul.kelly@anu.edu.au
- Dr Nick Anstey, Nicholas.anstey@menzies.edu.au

**Data and Safety Monitoring Committee:**
- Dr Paulus Sugiarto MD, Chair. 0811490658, paulus_sugiarto@hotmail.com
- Dr Louise Maple-Brown, Louise.Maple-Brown@menzies.edu.au
Adverse Events Definitions

Adverse Event
- Any adverse medical occurrence in an AVDAPT participant. The adverse event may or may not be due to the study medications. It includes symptoms reported by study subjects, signs noted by RA, or out-of-range laboratory values, which were not noted at baseline.

Serious Adverse Event
- Death
- Life-threatening condition
- Hospitalisation

Expected
The following are expected (recognized potential adverse effects of L-arginine and / or vitamin D):

- **L-arginine:**
  - Diarrhoea
  - Bloating
  - Nausea, Vomiting
  - Mild to moderate abdominal pain

- **Vitamin D:**
  - Asymptomatic hypercalcaemia defined as $iCa^{2+} > 1.32$ mmol/L
  - Symptomatic hypercalcaemia i.e. $iCa^{2+} > 1.32$ mmol/L with one or more of the following symptoms or signs:
    - Gastrointestinal: Nausea, Vomiting, Abdominal pain, Constipation, Anorexia
    - Neurological / Psychiatric: Fatigue, Irritability, Confusion, somnolence, Coma
    - Renal: Polyuria, dehydration
    - Others: Bone pain, itch.

Unexpected
Adverse events other than those listed above are considered to be unexpected.

Relationship to study drug

<table>
<thead>
<tr>
<th>Unrelated</th>
<th>Tidak berhubungan</th>
<th>Adverse event is clearly due to extraneous causes (e.g., underlying disease, environment)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Unlikely (must have 2)</td>
<td>Tidak</td>
<td>1. Timing of adverse event did not fit with start / finish of study drug</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2. Adverse event could have been due to TB</td>
</tr>
<tr>
<td></td>
<td></td>
<td>3. Adverse event could be due to something else such as TB medications or external (environmental) factor.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>4. Adverse event is not a recognized possible response to the medication.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5. Does not reappear or worsens with reintroduction of intervention</td>
</tr>
</tbody>
</table>

| Possible (must have 2) | Mungkin | 1. Timing of adverse event does fit with start / finish of study drug |
|                       |         | 2. Adverse event not due to TB |
|                       |         | 3. Adverse event not due to something else such as TB medications or external (environmental) factor. |
|                       |         | 4. Adverse event is a recognized possible response to the medication. |

| Probable (must have 3) | Dicurigai | 1. Timing of adverse event does fit with start / finish of study drug |
|                       |          | 2. Adverse event not due to TB, TB medications or external (environmental) factor. |
|                       |          | 3. Adverse event is a recognized possible response to the medication. |
|                       |          | 4. Adverse event disappears or decreases when study drug reduced or stopped. |

| Definite (must have all 4) | Ya | 1. Adverse event started after drug introduced |
|                          |    | 2. Adverse event not due to TB, TB medications or external (environmental) factor. |
|                          |    | 3. Adverse event is a recognized possible response to the medication. |
|                          |    | 4. Adverse event disappears or decreases when study drug reduced or stopped, and recurs with re-exposure. |
# FORMULIR AKIBAT SAMPINGAN SERIUS (ASS)

Serious Adverse Events Reporting Form

<table>
<thead>
<tr>
<th>Nama peserta [pertama]:</th>
<th>[keluarga]:</th>
<th>Tanggal SAE:</th>
<th>Code: TAD-</th>
</tr>
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<tbody>
<tr>
<td>Name</td>
<td>Surname</td>
<td>Date of SAE</td>
<td>2015.10</td>
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<table>
<thead>
<tr>
<th>Tempat:</th>
<th>Name pewawancara:</th>
</tr>
</thead>
<tbody>
<tr>
<td>TB Clinic</td>
<td>_______________________</td>
</tr>
<tr>
<td>RSMM</td>
<td></td>
</tr>
<tr>
<td>Puskesmas</td>
<td></td>
</tr>
<tr>
<td>Lain</td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Jenis akibat sampingan serius (ASS)</th>
<th>Nama pewawancara:</th>
</tr>
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<tbody>
<tr>
<td>Fatal</td>
<td></td>
</tr>
<tr>
<td>Mengancam jiwa</td>
<td></td>
</tr>
<tr>
<td>Rumah sakit</td>
<td></td>
</tr>
<tr>
<td>Lain</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Kesudahan</th>
<th>Nama pewawancara:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sembuh</td>
<td></td>
</tr>
<tr>
<td>Terus berlangsung</td>
<td></td>
</tr>
<tr>
<td>Meninggal</td>
<td></td>
</tr>
<tr>
<td>Tidak tahu</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>SAE type</th>
<th>Kesudahan</th>
<th>Nama pewawancara:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fatal</td>
<td>Sembuh</td>
<td></td>
</tr>
<tr>
<td>Life-threat</td>
<td>Terus berlangsung</td>
<td></td>
</tr>
<tr>
<td>Hosp</td>
<td>Meninggal</td>
<td></td>
</tr>
<tr>
<td>Death</td>
<td>Tidak tahu</td>
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</tr>
</tbody>
</table>

<table>
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<th>Tanggal Kesudahan</th>
<th>Nama pewawancara:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Hubungan dengan obat</th>
<th>Tidak berhubungan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Causal relationship to study drug</td>
<td>1 Unrelated</td>
</tr>
<tr>
<td>Tidak</td>
<td>2 Unlikely</td>
</tr>
<tr>
<td>Mungkin</td>
<td>3 Possible</td>
</tr>
<tr>
<td>Dicurigal</td>
<td>4 Probably</td>
</tr>
<tr>
<td>Ya</td>
<td>5 Definite</td>
</tr>
</tbody>
</table>
NIHRD-MSHR Health Research Collaboration ADAPT study  
Study HREC Number 07/40

ADAPT SAE Report Form  
Code: TAD-

<table>
<thead>
<tr>
<th>Informasi obat penelitian:</th>
<th>1. Arginine / Arginine placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tanggal mulai</td>
<td><strong>/</strong>/20__</td>
</tr>
<tr>
<td>Start date</td>
<td></td>
</tr>
<tr>
<td>Berapa bungkus yang tidak diminum?</td>
<td>at least 1__</td>
</tr>
<tr>
<td>How many doses were missed?</td>
<td></td>
</tr>
<tr>
<td>Perubahan dosis obat</td>
<td>Tidak 1 No</td>
</tr>
<tr>
<td>penelitian?</td>
<td>Dikurangi 2 Reduced</td>
</tr>
<tr>
<td>Study drug dose changed?</td>
<td>Stop 3 Stopped</td>
</tr>
<tr>
<td>Diberikan kembali</td>
<td>Ya 1</td>
</tr>
<tr>
<td>rechallenged?</td>
<td>Tidak 0</td>
</tr>
<tr>
<td>Tanggal diberikan kembali</td>
<td><strong>/</strong>/20__</td>
</tr>
<tr>
<td>Date rechallenged</td>
<td></td>
</tr>
<tr>
<td>Apakah ASS terjandi kembali?</td>
<td>Ya 1</td>
</tr>
<tr>
<td>Did SAE reappear?</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Informasi obat penelitian:</th>
<th>2. Vitamin D / Vitamin D placebo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Waktu pertama minum Vitamin D / Vitamin D placebo?</td>
<td>Ya 1 Tanggal: <strong>/</strong>/20__</td>
</tr>
<tr>
<td>Was the first vitD/plac dose taken?</td>
<td>Tidak 0</td>
</tr>
<tr>
<td>Waktu kedua minum Vitamin D / Vitamin D placebo?</td>
<td>Ya 1 Tanggal: <strong>/</strong>/20__</td>
</tr>
<tr>
<td>Was the second vitD/plac dose taken?</td>
<td>Tidak 0</td>
</tr>
<tr>
<td>Hypercalcaemia?</td>
<td>Ya 1</td>
</tr>
<tr>
<td>Did SAE reappear?</td>
<td>Tidak 0</td>
</tr>
<tr>
<td>Perubahan dosis obat</td>
<td>Tidak 1 No</td>
</tr>
<tr>
<td>penelitian?</td>
<td>Withheld 2 Withheld</td>
</tr>
<tr>
<td>Study drug dose changed?</td>
<td>Stop 3 Stopped</td>
</tr>
<tr>
<td>Diberikan kembali</td>
<td>Ya 1</td>
</tr>
<tr>
<td>rechallenged?</td>
<td>Tidak 0</td>
</tr>
<tr>
<td>Tanggal diberikan kembali</td>
<td><strong>/</strong>/20__</td>
</tr>
<tr>
<td>Date rechallenged</td>
<td></td>
</tr>
<tr>
<td>Apakah ASS terjandi kembali?</td>
<td>Ya 1</td>
</tr>
</tbody>
</table>
## AVDAPT AE Report Form

### Apakah Investigator melihat kode studi?
- Ya: [ ]
- Tidak: [ ]

### Has investigator unblinded treatment?
- Yes: [Y] [ ]
- No: [N] [ ]

### Peserta grup pengpbatan?
- Active Arg / Active VitD: [ ] [ ]
- Placebo Arg / Active VitD: [ ] [ ]
- Active Arg / Placebo VitD: [ ] [ ]
- Placebo Arg / Placebo VitD: [ ] [ ]

### Code: TAD- [ ] [ ] [ ] [ ]

<table>
<thead>
<tr>
<th>Obat lain</th>
<th>Ya</th>
<th>Obat</th>
<th>Tanggal mulai</th>
<th>Tanggal berakhir</th>
</tr>
</thead>
<tbody>
<tr>
<td>Other meds</td>
<td>[ ]</td>
<td>[ ]</td>
<td>[ ]</td>
<td>[ ]</td>
</tr>
</tbody>
</table>

### Catatan; Riwayat penyakit yang berhubungan

Notes, relevant medical history

---

After completing adverse event form, notify Dr Anna Ralph anna.ralph@anu.edu.au and Dr Dina Lolong dina_lolong@litbang.depkes.go.id
15.11 HYPERCALCAEMIA MANAGEMENT GUIDELINE

Hypercalcaemia in AVDAPT patients

- Any patient found to have ionized calcium > 1.32 for the first time should have the test repeated.
- If iCa > 1.32 mmol/L at baseline, patient not eligible for enrolment in the study.
- If iCa > 1.32 mmol/L at any reading during treatment, follow the flow chart below.
- Hospitalization should be avoided where possible (IV saline can be given in the TB clinic or outpatient clinic); requirement for hospitalisation in severe, symptomatic people will be discussed on a case-by-case basis.

<table>
<thead>
<tr>
<th>Hypercalcaemia Grade</th>
<th>Ionised calcium</th>
<th>Equivalent total calcium level (=iCax2)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mild</td>
<td>1.33 – 1.39 mmol/L</td>
<td>2.60 – 2.79 mmol/L</td>
</tr>
<tr>
<td>Moderate</td>
<td>1.40 – 1.49 mmol/L</td>
<td>2.80 – 2.99 mmol/L</td>
</tr>
<tr>
<td>Severe, potentially symptomatic</td>
<td>1.50 – 2.25 mmol/L</td>
<td>3.00 – 4.49 mmol/L</td>
</tr>
<tr>
<td>Very severe</td>
<td>&gt; 2.25 mmol/L</td>
<td>&gt; 4.50 mmol/L</td>
</tr>
</tbody>
</table>

**Symptomatic hypercalcaemia** will be defined as one or more of the following symptoms or signs occurring in a person with ionised calcium > 1.5 mmol/L
1. Gastrointestinal: Nausea, Vomiting, Abdominal pain, Constipation, Anorexia
2. Neurological / Psychiatric: Fatigue, Irritability, Confusion, somnolence, Coma
3. Renal: Polyuria, dehydration
iCa > 1.32 mmol/L

- **Mild**
  1.32 – 1.39
  - Observe and repeat blood test within next week

- **Moderate**
  1.40 – 1.49
  - Increase oral fluid intake. Observe and repeat test within 3 days

- **Severe**
  >1.50
  - **Asymptomatic**
    - Treat with IV saline +/- frusemide
  - **Symptomatic**
    - Treat with IV saline and oral prednisolone 15-60mg/day
15.12 EXAMPLE OF EPIDATA DATABASE

1. ‘QES File’ written to generate data entry program for Enrolment questionnaire

DATA DASAR

| STUDY  | <AAA>               |
| CODE   | # # #               |
| ID     | <AAAAAAA>           |

DATE0 Enrolment Date @<dd/mm/yyyy>

Bagain A: Kriteria inklusi dan eksklusi

NAMAP __________________________

NAMAF __________________________

RA #

Inclusion:
SMPOS #

TBNEW #

STAYTIM #

ADULT #

CONSENT #

Exclusion:

PREG #

CALHI #

VIOLATION #

Bagain B: Informasi dasar peserta

SITE #

DOB <dd/mm/yyyy> #

AGE #

ADDRESS _________________________

PHONE #

SEX #

PAPUAN <AAAAAAAAAAAAA>

SUKU #

EDUC #

EMPLOY1 #

EMPLOY2 #

SMOK #

SMOKNO #

ILLTIME #.##

HEIGHT #.##

RELATE TO OTHER DATABASES

To jump to other database, place cursor in box and press return. To get back to the BASELINE file press F10

RELATE #

2. ‘CHECK file’ then applied to provide labels, calculations, internal consistency checks etc

3. ‘REC file’: Data entry screen

3. View data screen: creates spreadsheet
Understanding how TB causes lung disease

We would like to ask you to take part in a study testing how far you can walk in 6 minutes and how TB causes lung damage. You are suitable for this study because you are healthy. We want to compare your results with people with tuberculosis (TB), a lung illness which affects breathing. To find out how their lungs compare with normal, we need to test healthy people to see how far they can walk, what their breathing tests show, and how well their blood may fight infection.

If you want to take part, we will ask you a few questions about your health, check how much you weigh, test your breathing (exhaled nitric oxide) then ask you to walk along the marked track for 6 minutes. We will also ask you for a small amount of blood: 20 ml (about 4 teaspoons): this will be used to examine the ability of healthy people to fight infection, so we can compare this to people with TB. These tests will not cost you anything.

All information collected is confidential. No personal identification will be revealed to persons outside the study.

You do not have to participate if you don’t want to, this is entirely voluntary.

These tests will not benefit you directly. But by helping us better understand how TB causes disease, we may be better able to prevent and treat TB in the future.

If you have any questions about the study you can telephone Dr Paulus Sugiarto at RS Mitra Masyarakat Hospital on 901-301 881. If you have any concerns or complaints about the conduct of the study, please contact Dr Sandjaja, Chair of the NIHRD Ethics Committee in Jakarta on 081319304040 or 6202104261088, or The Secretary, Human Research Ethics Committee of the NT Department of Health & Community services & Menzies School of Health Research in Australia on 61-8-8922 7922
Understanding how TB causes lung disease

This form means you can say No.

I have read the information sheet and have had the details explained to me by the witness below. I understand that I am not sick and will only need to answer questions, have my weight measured, a blood test (20 mls = 4 teaspoons), and walk for 6 minutes. I understand that all information collected is confidential and no information will be available to anyone outside the study. I understand that I do not have to participate in this study. I can withdraw from this study at any time for any reason.

PARTICIPANT
I (print name)  
…………………………………………………………... agreeto take part in this study.  
Signed  
…………………………………………………………... Date  
…………………………………………………………... _____ / _______ / 20_____

PARTICIPANTS AGED <18 YEARS: PARENT / GUARDIAN TO SIGN
I (print name)  
…………………………………………………………... agree for my child / person in my care to take part in this study.

Participant name  
…………………………………………………………...  
Signed  
…………………………………………………………... Date  
…………………………………………………………... _____ / _______ / 20_____

WITNESS
I (print name)  
…………………………………………………………... have explained the study & information sheet  
Signed  
…………………………………………………………... Date  
…………………………………………………………... _____ / _______ / 20_____

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AVDAPT Controls  
Code THC-  

15.14 HEALTHY VOLUNTEER DATA COLLECTION FORM

FORMULIR PENGUMPULAN DATA ORANG SEHAT
Healthy people data collection form

Bagian A : Nama Peserta dan Pewawancara
Section A: Study Participant and data collector name

1. Tanggal hari:  
Date today

2. Nama [pertama]: ____________________________________________
First Name

3. Nama [keluarga]: _____________________________________________
Surname

4. Nama pengumpul data : __________________________________________
Data collector

Bagian B: Kriteria Inklusi dan Eksklusi
Section B: Inclusion and Exclusion Criteria

8. Umur > 15 th:  
Age >15yrs

9. Pulse <120/min?

10. BP<180/100?

11. Persetujuan Consent untuk berpartisipasi dalam penelitian ini:
Consent given

Bila menjawab “tidak” untuk pertanyaan no. 5-8, pasien tidak bisa dimasukkan dalam penelitian ini. If ‘No’ to any questions 5-8, PATIENT IS NOT ELIGIBLE

12. Currently sick? (febrile illness or diagnosed by doctor with illness during the last week)?
Currently sick?

10.Unstable angina present?

11. Cough present? (only tick ‘ja’ if severe or new. Mild stable cough permitted)
Cough present?

Bila pasien menjawab “Ya” untuk pertanyaan no. 9-11, pasien tidak bisa diikutkan dalam penelitian ini. If ‘Yes’ to question 9-11, PATIENT IS NOT ELIGIBLE

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Bagian C : Data Peserta
Section C: Study Participant data

Alamat: ________________________________________________
Address

Telepon: ________________________________
Phone

Tanggal kelahiran: ______ / ______ / 19____
Date of birth: dd/mm/yyyy

Umur: ______ tahun
Age ______ yr

Jenis Kelamin:  Laki □1  Perempuan □2
Sex: M1,F2

Suku:  Papuan □1  Bukan Papuan □2
Ethnicity

Agats □1  Ekari □9  Merauke □17  Lain □25
Amungme □2  Fak-Fak □10  Moni □18
Asmat □3  Genyem □11  Nabire □19
Ayamaru □4  Jayapura □12  Nduga □20
Biak □5  Kamoro □13  NonPapuan □21
Bintuni □6  Damal □14  Paniai □22
Dani □7  Dani □15  Sarui □23
Danie □8  Mee □16  Sorong □24

Pendidikan:
Education: (Primary1, High2, Further3, nil4)

Sekolah Dasar □1  SMP/SMA □2
Akademi/Universitas □3  Tidak sekolah □4

Pekerjaan:
Employment (work, school, nil)

Bekerja □1 sebutkan_____________________
Pergi ke sekolah □2  Tidak ada □3

Riwayat merokok?
Smoker?

Merokok □1  Pernah merokok tetapi sudah berhenti □2
Tidak pernah merokok □3

Jika merokok, cigs/day?
Berapa banyak rokok yang diisap per hari?

_____/ hari
Bagian D: Clinical and laboratory measurements

**Jam berapa makan terakhir?**

How long ago did patient last eat? (hrs)

- **Pulse**
  - _______ bpm

- **Systolic BP**
  - _______ mmHg

- **Diastolic BP**
  - _______ mmHg

- **Hb**
  - _______ g/dL

- **Blood collected for lab**
  - Ya [ ]
  - Tidak [ ]

- **eNO**
  - _______ ppb

- **Lung function: FEV1**
  - _______ L/s

- **Lung function: FVC**
  - _______ L

- **Berat badan**
  - _______ kg

- **Height**
  - _______ m

- **Tes jalan 6 menit**
  - _______ m

- **Kuisioner Respiratori St George**
  - Ya [ ]
  - Tidak [ ]

Investigator name and signature: ____________________________________________