The immunomodulatory role of prednisone in patients with HIV-associated tuberculosis and assessment of its impact on lung function outcome

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Stek C, Buyze J, Menten J, Schutz C, Thienemann F, Blumenthal L, Maartens G, Boyles T, Wilkinson RJ, Meintjes G, Lynen L. *Diagnostic accuracy of the INSHI consensus case definition for the diagnosis of paradoxical tuberculosis-IRIS*. J Acquir Immune Defic Syndr. 2020 Dec 17; Publish Ahead of Print

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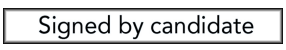


Abstract

In this thesis, I assess the effect of prednisone on immunopathology and pulmonary function in HIV-associated tuberculosis and tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS). I do this using data collected during the PredART trial. This randomized, double-blind, placebo-controlled trial showed that a 28-day course of prophylactic prednisone in adult patients identified as being at high risk for paradoxical TB-IRIS reduced the incidence of paradoxical TB-IRIS by 30%, without an excess of adverse events.

Chapter one

In this introductory chapter, I review general aspects of HIV, tuberculosis, and HIV-associated tuberculosis. Thereafter, I focus on paradoxical TB-IRIS, with an emphasis on what is known about its immunology. I discuss the use of prednisone in the treatment of tuberculosis and the possible effect of leukotriene A4 hydrolase (LTA4H). I review lung function impairment in tuberculosis and TB-IRIS. Since all data used in this thesis were collected during conduct of the PredART trial, as substudies that I conducted, I provide background to this trial in the introduction.

Chapter two

The diagnosis of paradoxical TB-IRIS relies on characteristic clinical features that have been synthesized by the International Network for the Study of HIV-associated IRIS (INSHI) as a case definition. There is no confirmatory laboratory test. In chapter two, I report a study in which I applied latent class analysis to model a potential gold standard case definition for TB-IRIS, using data from 217 participants in the PredART trial. I used the model-predicted probability of TB-IRIS for each participant to assess the performance of the INSHI case definition and compare its diagnostic accuracy with several adapted case definitions. I showed that the INSHI case definition identifies TB-IRIS with a sensitivity of 0.77 and a specificity of 0.86. Amending the case definition by replacing INSHI minor criteria with objective variables heart rate, temperature, and/or C-reactive protein improved its sensitivity without loss in specificity. CRP appears to be promising as a test for ruling out TB-IRIS.

Chapter three

In chapter three, I report a study in which I investigated the associations between cytokine/chemokine profiles, LTA4H genotype, development of TB-IRIS and use of prophylactic prednisone. I used stored plasma from 183 PredART participants, taken at baseline (start of antiretroviral therapy (ART)) and 2 weeks after the start of ART to measure levels of 31 cytokines and chemokines. I determined *lta4h* genotype of 213 PredART participants using DNA extracted from stored blood. I did not find a difference in cytokine profiles at baseline between participants who later developed TB-IRIS vs those who did not. TB-IRIS was associated with an increase in cytokines associated with both the innate and the adaptive immune system at week 2. Prednisone prophylaxis resulted in a decrease of these cytokines. *lta4h* genotype associated with neither the incidence nor the cytokine profile of TB-IRIS. I was unable to show that the efficacy of prednisone to prevent TB-IRIS is genotype dependent.

Chapter four

Chapter four provides a comprehensive review of mechanisms of lung damage in tuberculosis and potential strategies to prevent it. I first review the literature that has characterised lung damage in human tuberculosis. Next, I give an overview of the different immune pathways involved in lung damage in tuberculosis, once again focusing on findings in human tuberculosis and highlighting some of the challenges related to research in this area. Based on these findings, I discuss the potential of certain registered medications to serve as host-directed therapies to reduce lung damage and review the available evidence for their use. This chapter serves as an introduction to chapter five, in which I describe a lung function substudy conducted within the PredART trial.

Chapter five

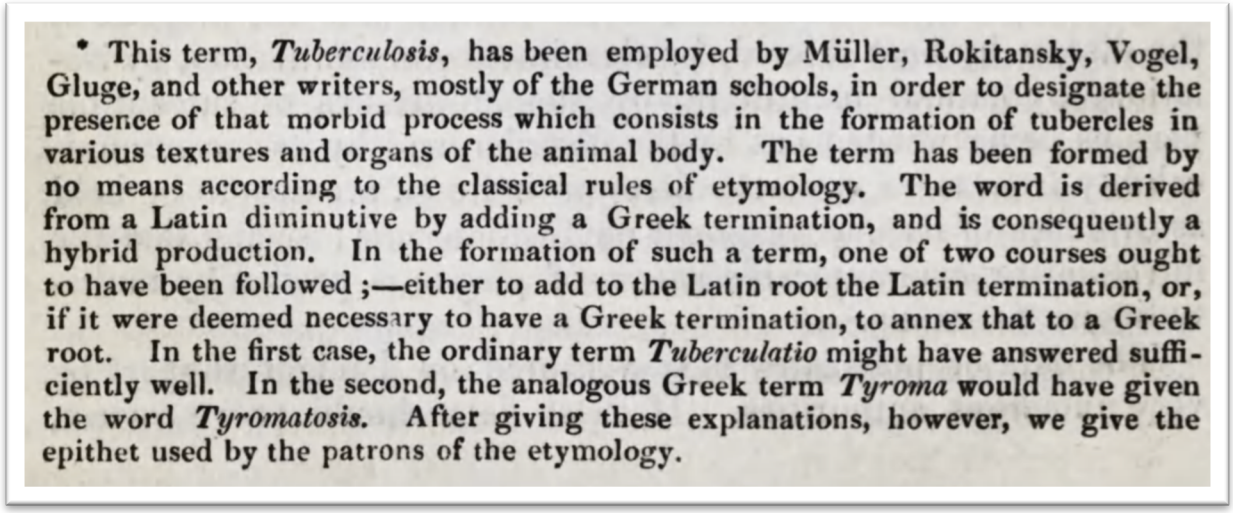
Chapter five focusses on study of lung function impairment during and after completion of treatment for HIV-associated tuberculosis. I determined the prevalence of lung function abnormalities in patients with HIV-associated tuberculosis and CD4 counts < 100 cells/µl and assessed the effect of prophylactic prednisone and the development of paradoxical TB-IRIS on lung function impairment. I performed spirometry, six-minute walk test, and chest radiography at baseline (week 0), week 4, 12, and 28 in 153 participants of the PredART trial. I found residual pulmonary impairment to be common in HIV-associated tuberculosis but did not find an effect of either prophylactic prednisone as used in our study or the development of TB-IRIS on long term pulmonary outcomes.

Chapter six

In chapter six I summarize the findings of the studies, draw broad conclusions from across the studies presented and provide recommendations for future research in these areas.

Introduction

Tuberculosis (TB) has been present throughout known human history and *Mycobacterium tuberculosis* may have killed more persons than any other microbe. Early forms of *Mycobacterium tuberculosis* were already present in East Africa 15,000 or more years ago [1]. In the centuries that followed, tuberculosis spread around the world. It has been found in Egyptian and Peruvian mummies, dating back to 2400 BC [2, 3] and was first described in reports originating from India and China, between 3300 and 2300 years ago. Tuberculosis was recognized as "phthisis" in ancient Greece, "tabes" in ancient Rome, and "schachepheth" in ancient Hebrew. It was widespread in Europe in the 17th and 18th century, known as "the white plague" and "the captain among these men of death", causing up to 25% of all deaths. In 1843 Johann Schonlein gave tuberculosis its current name.



* This term, ***Tuberculosis***, has been employed by Müller, Rokitansky, Vogel, Gluge, and other writers, mostly of the German schools, in order to designate the presence of that morbid process which consists in the formation of tubercles in various textures and organs of the animal body. The term has been formed by no means according to the classical rules of etymology. The word is derived from a Latin diminutive by adding a Greek termination, and is consequently a hybrid production. In the formation of such a term, one of two courses ought to have been followed ;—either to add to the Latin root the Latin termination, or, if it were deemed necessary to have a Greek termination, to annex that to a Greek root. In the first case, the ordinary term ***Tuberculatio*** might have answered sufficiently well. In the second, the analogous Greek term ***Tyroma*** would have given the word ***Tyromatosis.*** After giving these explanations, however, we give the epithet used by the patrons of the etymology.

Footnote to *Physiological-Pathological Researches on Tuberculosis* by Dr. H. Lebert, Edingb Med Surg J 1848

Less is known about the early history of tuberculosis in South Africa. Some speculate the Dutch settlers introduced the disease to the Cape in 1652. Outbreaks occurred but were isolated and the disease remained rare. It was not until the end of the 19th century that tuberculosis began to spread more rapidly: the favorable climate of South Africa attracted people suffering from tuberculosis-burdened Europe and the discovery of diamonds and gold led to rapid urbanization.

In 1904 tuberculosis was made a notifiable disease in the Cape. Tuberculosis notifications in Cape Town alone ranged between 350 - 550 / 100,000 people per year between 1910 and 1945. They decreased after the introduction of antituberculosis therapy, but never got below 250 / 100,000 people per year [4].

HIV has a much shorter history than tuberculosis. It became known to the world in 1981, when the first AIDS cases were reported among men who have sex with men in Los Angeles and New York [5]. However, it is believed that HIV originates from Kinshasa in the Democratic Republic of Congo around 1920 and spread from there to the rest of the continent and the world [6]. In South Africa, the first two described patients with HIV date from 1982 [7]; both were men who have sex with men who had recently spent time in the US. The incidence of HIV rose sharply in the following years [8] but the epidemic was largely confined to men having sex with men, foreign mine workers and patients with hemophilia. In the early 1990s however, heterosexual and mother-to-child transmission took over as the main transmission routes. HIV incidence kept rising until an estimated 550,000 new cases per year at the end of last century [9]. Currently, South Africa is the country with the highest numbers of people with HIV: of the almost 38 million people living with HIV at the end of 2018 worldwide [10], 7.7 million (20%) live in South Africa [9].

The HIV epidemic is a major factor driving the tuberculosis epidemic in South Africa: the incidence of tuberculosis (in Cape Town) doubled between 1990 and 2010 [4]. Tuberculosis incidence in South Africa is estimated at 519 / 100,00 people in 2018, also one of the highest in the world [11] .

HIV-associated tuberculosis

Globally, of the estimated 10 million people who fell sick with tuberculosis in 2018, 9% were HIV positive [11]. In South Africa, however, the HIV-tuberculosis co-infection rate is as high as 60%. Diagnosis of tuberculosis in people with HIV remains difficult because of the often-atypical presentation and the low mycobacterial load in sputum. At the same time, tuberculosis progresses more rapidly in the presence of HIV co-infection and remains the leading cause of death in this patient population. Treatment of HIV-associated tuberculosis poses several challenges, including drug-drug interactions, shared side effects of medication and paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS). The first two are beyond the scope of this introduction, the latter forms the topic of this thesis. Initially, these factors caused clinicians to delay antiretroviral therapy in patients on treatment for tuberculosis. However, several clinical trials in the past 15 years have shown that early ART initiation (within 4 weeks after starting antituberculosis therapy) reduced mortality by about 30% compared with delayed ART (8-12 weeks after starting antituberculosis treatment) in patients with a low CD4 count [12, 13]. Therefore, ART initiation should not be delayed in patients with a CD4-count less than 50/µl. However, this increases the risk of TB-IRIS.

People with HIV have an increased risk of developing tuberculosis disease. The most obvious reason for this is a decrease in CD4 cells. CD4+ T-cells and their cytokines (TNF-α and IFN-γ)

are essential in controlling TB, as demonstrated by animal studies in mice lacking IFN-γ or TNF-α and macaques with depleted T-cells [14-16]. CD4+ T-cells play a role in activating macrophages as well as in the formation and function of granuloma. However, HIV-co-infection already increases the risk of TB even before CD4 counts have dropped profoundly. Possibly, early HIV infection specifically depletes *Mtb*-specific CD4+ T-cells due to their high expression of CCR5 or alters T-cell phenotype to generate a suboptimal response. Alternatively, HIV affects the innate immune response. HIV affects neutrophil function and inhibits macrophage apoptosis in response to *Mtb* infection and could affect antigen presentation by dendritic cells [17].
Mtb infection also affects the course of HIV. Proinflammatory cytokine production by innate immune cells and activation of macrophages in response to *Mtb* may increase HIV replication and progression of disease [17].

Paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome

> "I was told that it's a virus that occurs when you are eating ARVs and you combine them with TB treatment. They react when they come together. So, this is the virus that came out as a result of the weakening of one's immune system."

Participant SN255, when she was asked to explain what TB-IRIS is.

Incidence and risk factors of TB-IRIS

A systematic meta-analysis in 2015 showed paradoxical TB-IRIS complicates treatment of HIV-associated tuberculosis in 18% (95% CI 16-21%) [18]. More recent cohorts prospectively following adult patients with HIV-associated tuberculosis starting ART find similar incidences of TB-IRIS between 9.8% and 59% [19-22]. However, one study in which 20 patients with active tuberculosis starting ART were followed prospectively in Gabon found no cases of TB-IRIS [23].

There are several risk factors for TB-IRIS. Low CD4 count and high viral load are identified in several prospective studies, including randomized controlled trials, although in two more recent prospective cohorts [19, 21] baseline CD4 count did not differ between patients who did or did not develop TB-IRIS. A short interval between the start of antituberculosis therapy and ART is another well-established risk factor: the risk of TB-IRIS more than doubles in patients starting ART early (up to 4 weeks) compared to those starting late (8-12 weeks). These findings come from two meta-analyses of randomized controlled trials on the optimal time to start ART after initiation of anti-tuberculosis therapy in patients with HIV- associated tuberculosis that report combined relative risks of TB-IRIS of 2.19 (95% CI 1.77-2.20) and 2.31 (95% CI 1.82-2.86) for early versus late ART initiation [12, 13]. High mycobacterial load,

associated with extrapulmonary tuberculosis and reflected in a positive urine lipoarabinomannan (LAM), is also associated with TB-IRIS [18, 24-26].

Diagnosis and treatment of TB-IRIS

TB-IRIS presents as new, recurrent, or worsening signs or symptoms of TB. The most commonly reported features are fever; respiratory symptoms, like cough or shortness of breath; and lymph node involvement. Neurological features or serositis are less common. TB-IRIS typically presents within the first four weeks after starting ART but has been recorded to occur after three months or even longer. Symptoms last on average 1-3 months, but mild cases lasting only a few days as well as prolonged clinical courses (for more than one year) have been described [18, 27], the latter especially in patients with lymph node involvement [28]. TB-IRIS requires hospitalization in 25% of the patients. Mortality is uncommon (2%) [18], although central nervous system TB-IRIS carries a much higher mortality risk (13%) [29, 30].

The diagnosis of TB-IRIS is largely based on its characteristic clinical presentation; there is no confirmatory laboratory test. The International Network for the Study of HIV-associated IRIS (INSHI) consensus case definition is used to diagnose TB-IRIS [27]. It requires a diagnosis of tuberculosis with an initial positive response to treatment, characteristic clinical features (such as new or enlarging lymphadenopathy, constitutional, respiratory or abdominal symptoms, or new or worsening radiological features), and exclusion of alternative explanations for clinical deterioration and is summarized in Panel 1.1. It is designed for use by both clinicians and researchers in a variety of settings and has been validated in several studies [31-33].

Ever since the first reports on paradoxical worsening of tuberculosis after starting ART in the late 1990s, treatment of TB-IRIS with anti-inflammatory therapy like prednisolone [34] or oxpentifylline [35] has been taking place and administration of short-term steroids that suppress the enhanced immune response while continuing antituberculosis therapy and ART has been recommended [36]. However, it was not until 12 years later that the efficacy of prednisone to treat TB-IRIS was supported by data from a randomized controlled trial [37]: treatment with prednisone (1,5 mg/kg for 2 weeks, followed by 0.75 mg/kg for 2 weeks) was shown to reduce symptoms and the need for hospitalization and interventions.

Other anti-inflammatory drugs have been used to treat TB-IRIS. Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the conversion of arachidonic acid into pro-inflammatory prostaglandins. Their use has been described in TB-IRIS [38-40], but scientific evidence for efficacy is lacking. There are case reports describing successful treatment of TB-IRIS in individual patients with the TNF-α inhibitor infliximab [41, 42]; clinical trials are needed before we can conclude these drugs are effective and safe in patients with HIV-associated tuberculosis.

(A) Antecedent requirements
Both of the two following requirements must be met:

- Diagnosis of tuberculosis
- Initial response to tuberculosis treatment

(B) Clinical criteria
The onset of TB-associated IRIS manifestations should be within 3 months of ART (re)initiation or regimen change because of treatment failure. Of the following, at least one major or two minor criteria are required:
Major criteria

- New or enlarging lymph nodes, cold abscesses, or other focal tissue involvement
- New or worsening radiological features of tuberculosis
- New or worsening central nervous system tuberculosis
- New or worsening serositis

Minor criteria

- New or worsening constitutional symptoms such as fever, night sweats or weight loss
- New or worsening respiratory symptoms such as cough, dyspnea, or stridor
- New or worsening abdominal pain accompanied by peritonitis, hepatomegaly, splenomegaly or abdominal adenopathy

(C) Alternative explanations for clinical deterioration must be excluded if possible

- Failure of tuberculosis treatment because of tuberculosis drug resistance
- Poor adherence to tuberculosis treatment
- Another opportunistic infection or neoplasm
- Drug toxicity or reaction

PANEL 1.1 - INSHI case definition for paradoxical tuberculosis associated IRIS [27]

Two double-blind randomized, placebo-controlled studies have assessed strategies to prevent TB-IRIS in adults. Both studies included ART naïve patients with a CD4 count ≤100 cells/µl; the diagnosis of IRIS was adjudicated by independent committees. The CADIRIS (CCR5 Antagonism to Decrease the Incidence of the Immune Reconstitution Inflammatory Syndrome in HIV infected Patients) trial [22] assessed the efficacy of the CCR5 blocker maraviroc in reducing all IRIS, including TB-IRIS, with time to an IRIS event by 24 weeks as the primary outcome. 276 participants were randomized to receive either maraviroc (600 mg twice daily) or placebo in addition to standard ART for 48 weeks. Sixty-four participants had a known tuberculosis infection at baseline. Seventeen participants developed paradoxical TB-IRIS; no difference in proportion of TB-IRIS was found between the maraviroc and the placebo arm. The PredART trial [43] assessed the efficacy and safety of prophylactic prednisone in preventing TB-IRIS in patients who are identified as being at high risk for paradoxical TB-IRIS. This trial forms the context of this thesis and will be discussed in detail below.

Pathogenesis of TB-IRIS

French et al describe in 1990 that zidovudine therapy in patients with HIV restores cutaneous delayed-type hypersensitivity reactions against mycobacterial antigens, “which in some cases is so extreme that a ‘hypersensitivity reaction’ occurs, resulting in fevers and tissue inflammation” [44]. After the introduction of combination ART, more reports of

patients with paradoxical worsening of tuberculosis after starting ART were described [34, 35, 45]. The reactions coincided with improved CD4 lymphocyte counts, decreases in plasma HIV load, and strongly positive purified protein derivate test reactions. It was therefore hypothesized that restoration of mycobacteria specific T cell response played an important role in the pathogenesis of TB-IRIS. Indeed, it was shown interferon-γ (IFN-γ)-producing Th1 cell numbers increased sharply during paradoxical TB-IRIS [46-50]; especially effector memory-type Th1 cells [51, 52]. However, this expansion was not present in all IRIS patients and also occurred in patients without TB-IRIS [40, 47, 53, 54], making it unlikely the relation is causal. Other T-cells, like regulatory T cells, γδ T-cells and invariant natural killer T-cells have also been implicated in the pathogenesis of TB-IRIS, although findings differ between studies [47, 55-58].

A well characterized feature in TB-IRIS is hypercytokinemia [59]. Various studies found increased levels of chemo- and cytokines in plasma or supernatant of stimulated PBMCs [21, 48, 60-67]. Early reports suggest an imbalance between inflammatory and regulatory cytokines [68] could underlie IRIS. However, the immunoregulatory and anti-inflammatory interleukin (IL)-10 was also increased in many patients with TB-IRIS [69].

As many of the cytokines found to be increased are of myeloid origin [60, 66], more recently attention has shifted to the role of the innate immune system in TB-IRIS. Transcriptomic analysis of whole blood of patients with TB-IRIS indeed identified the differential expression of innate immune mediators including toll-like receptor signaling and inflammasome activation, compared with control patients . Inflammasomes are a group of intracellular multimeric protein complexes that assemble in response to pathogen-associated or danger-associated molecular patterns. Their activation has been associated with TB-IRIS [67, 70]. Their assembly leads to activation of caspases, which in turn generate the pro-inflammatory IL-1β and IL-18. They are predominantly present in monocytes and macrophages.

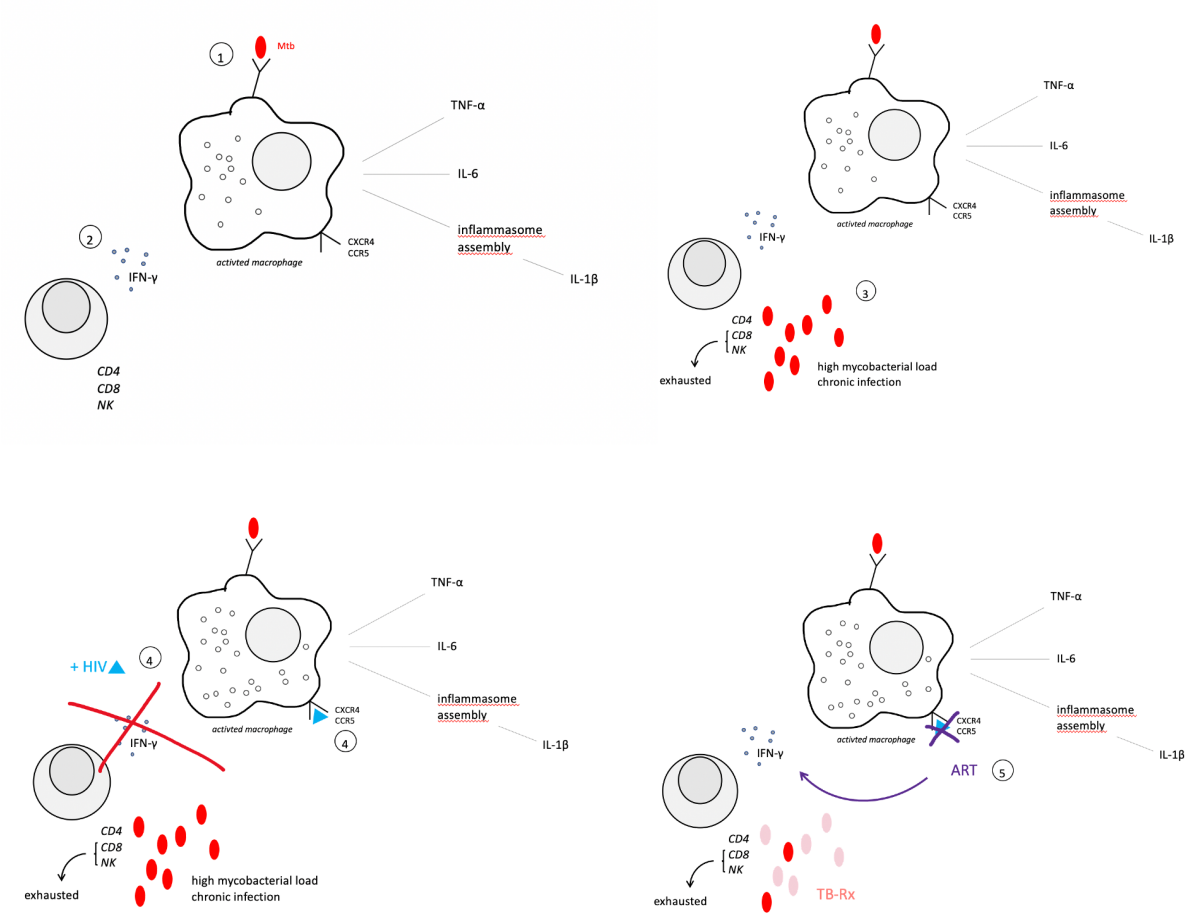


FIGURE 1.1 – Monocytes in TB-IRIS

For explanation, see text

It is hypothesized that monocyte activation may lead to hypercytokinemia and excess inflammation in TB-IRIS – see Figure 1.1. Classically activated monocytes/macrophages require two signals to become activated: recognition of the microorganism (1), in this case Mycobacterium tuberculosis, which primes the cells for further activation, followed by IFN-γ stimulation (2), needed to fully activate the cells. Both HIV and tuberculosis infection affect monocytes. Their function is dysregulated by CXCR4 and CCR5 receptor triggering on their surface by HIV viral antigens [71] and the absence of IFN-γ-producing CD4+ T-cells needed to fully activate macrophages (4), resulting in an uncoupling of the innate and the adaptive immune system [72]. Moreover, long-term tuberculosis infection and/or high mycobacterial load can lead to exhaustion of subsets of NK and CD8+ T cells (3), contributing to further lack of IFN-γ signaling [73]. When both infections occur simultaneously in one patient, *Mycobacterium tuberculosis* still primes macrophages, but activation is incomplete in the presence of HIV viral antigens and the lack of IFN-γ (4). ART lowers HIV viral load, restoring the monocyte dysregulation by HIV viral antigens (5); indeed, suppression of HIV viral load leads to more tumor necrosis factor (TNF)-α and IL-6 secretion (6) by monocytes [61]. Only a few CD4+ T-cells are then needed to fully activate monocytes. This hypothesis explains the

risk factors identified for TB-IRIS: low CD4 count, high HIV viral load, and high mycobacterial load and short interval between the start of antituberculosis treatment and the start of ART.

Other innate immune cells may also be involved: increased natural killer cell activation and degranulation activity have been associated with TB-IRIS [74, 75]; and neutrophils were found to be increased, activated [76] and present at the site of disease [76, 77]. Moreover, matrix metalloproteinases - a family of proteolytic enzymes whose primary function is degradation of the extracellular matrix, but are also involved in tissue repair, remodeling and modulation of the immune response – appear to play an important role in tissue damage in TB-IRIS [19, 78, 79], especially MMP-8.

Other forms of IRIS

Unmasking TB-IRIS occurs when TB was not diagnosed before ART initiation. Patients present with an acute inflammatory form of TB within 3 months after ART initiation. Unmasking TB-IRIS occurs in 1-6% of patients starting ART in sub-Saharan Africa. It presents with inflammatory TB symptoms, like lymphadenitis, weight loss, meningism, abscesses or respiratory failure. Risk factors are - like in paradoxical TB-IRIS - a low CD4 count and a high viral load. Moreover, signs of subclinical TB such as anemia, weight loss, and high CRP are predictive of developing unmasking IRIS. There are no specific therapeutic interventions for unmasking TB-IRIS. Guidelines advise continuation of ART; sometimes corticosteroids are used to control severe inflammatory manifestations [80].

Pathogens other than TB are also associated with IRIS. The most common ones are non-tuberculous mycobacteria, *Cryptococcus,* and various viruses.
Mycobacterium Avium (MAC) was responsible for IRIS in 7.3% of participants starting ART in a large multi-country prospective cohort study; is it likely to be lower in patients with a higher CD4 count. Most of these cases were unmasking MAC-IRIS, presenting with fever, lymphadenopathy, or pulmonary disease, on average 7-8 weeks after ART initiation. Treatment of MAC-IRIS consists of treating the underlying infection, possibly combined with corticosteroids [81].
Cryptococcal IRIS occurs in about 20% of patients on treatment for cryptococcal disease starting ART [82]. It presents on average 1-10 months after ART initiation as either worsening of previously diagnosed cryptococcal disease or as an unmasking IRIS. Most cases present with meningeal signs and symptoms, but other central nervous system manifestations, skin manifestations, lymphadenopathy and pulmonary disease can also occur [83]. There are no clear guidelines for treatment of cryptococcal IRIS, but management with therapeutic lumbar punctures and immunosuppressive treatment like NSAIDs and corticosteroids has been described [84].

Various viruses, like herpes simplex virus, varicella zoster virus and molluscum contagiosum virus - mostly related to mucocutaneous conditions - are associated with IRIS. Human herpes virus-8 (HHV-8) is the underlying cause of Kaposi sarcoma (KS). KS-IRIS occurs in 7-31% of patients starting ART, usually within the first 12 weeks. It presents with either enlargement of existing lesions or rapid development of new lesions. Treatment includes chemotherapy. ART should be continued; corticosteroids are contra-indicated [85].

Corticosteroids in tuberculosis

Corticosteroids have long been used as adjunctive treatment in tuberculosis, recognizing that the host immune response against *Mycobacterium tuberculosis* contributes to the pathology [86]. Their use is proven beneficial in the treatment of tuberculous meningitis (TBM) [87], pericardial tuberculosis [88], and paradoxical TB-IRIS [37]. For pulmonary tuberculosis, a Cochrane review including 18 studies found no evidence that corticosteroids affects all-cause mortality (RR 0.77, 95%CI 0.51 - 1.15) or sputum culture conversion rate (RR 1.03, 95%CI 0.97 - 1.09) [89]. However, in only five of these studies, rifampicin-containing tuberculosis treatment was used; only one study included HIV co-infected patients. In the latter study, sputum culture conversion rate was significantly faster in patients using prednisone during the first two months of antituberculosis treatment, compared to patients not using steroids [90]. This was in a group of patients not on ART, with CD4 counts above 200 cells/µl and an initial prednisone dose of 2.75 mg/kg/day. A meta-regression analysis, examining the relationship between corticosteroid dose and sputum culture conversion, found that adjunctive steroid treatment does accelerate sputum tuberculosis culture conversion; however high doses (134 mg prednisone daily) for an extended period (2 months) are required to reach clinically relevant outcomes [91]. The underlying mechanism is unclear. It has been suggested that corticosteroids may improve drug penetration into lung lesions and enhance antimicrobial drug action by promoting bacillary aerobic metabolic activity [92].

In tuberculous meningitis, a Cochrane review including nine studies conducted between 1969 and 2009 found corticosteroid use was associated with 25% fewer short- and medium-term deaths (RR 0.75, 95% CI 0.65 - 0.87) [87]. Of these nine trials, however, again only one study included patients co-infected with HIV [93]. Although not powered to assess efficacy in this subgroup of patients, the authors found no significant effect of dexamethasone on death or the combined endpoint of death and disability in HIV infected patients with tuberculous meningitis. Its safety and efficacy are currently being assessed in a randomized trial limited to HIV infected patients [94].

Pericardial tuberculosis has also been the topic of a recent Cochrane review [95]. Six studies conducted between 1959 and 2014 were included that assessed the effect of corticosteroids. The authors concluded that in patients not infected with HIV, corticosteroids may reduce all-cause mortality (RR 0.88, 95% CI 0.59 - 1.09) and death from

pericarditis (RR 0.39, 95% CI 0.19 - 0.80). Again, in patients co-infected with HIV they found no clear evidence for such effect of corticosteroids (RR 0.91, 95%CI 0.34 – 2.24 for all-cause mortality; RR 1.07, 95% CI 0.46 – 2.54 for death from pericarditis)); corticosteroids may reduce pericardial constriction in this group (RR 0.55, 95%CI 0.26 – 1.06). It must be noted that only 1 in 5 patients co-infected with HIV was on ART.
The role of corticosteroids in the treatment of TB-IRIS has been discussed above.

A recent study suggests that the efficacy of corticosteroids as adjuvant treatment in tuberculosis might depend on *lta4h* genotype [96]. Leukotriene A4 hydrolase (LTA4H) is an enzyme that hydrolyses leukotriene (LT) A4 to LTB4, a strong pro-inflammatory eicosanoid that attracts neutrophils and other immune cells and stimulates production of pro-inflammatory cytokines [97]. In zebrafish, both over- and underexpression of LTA4H negatively influences the outcome of mycobacterial infection: overexpression leads to increased levels of LTB4 resulting in initial improved mycobacterial control, followed by excessive TNF-α driven inflammation. Underexpression favors production of the anti-inflammatory lipoxane (LX) A4, resulting in down-modulation of the immune response necessary to control mycobacterial growth [96]. Both scenarios eventually result in necrosis of macrophages with release of mycobacteria into the extracellular environment. A single nucleotide polymorphism (SNP) close to the promotor region of the *lta4h* gene is described to regulate LTA4H activity in humans [98]. The wildtype genotype (CC) is associated with lower levels of LTA4H, whereas the double mutant genotype (TT) is associated with increased LTA4H activity. Several studies have evaluated the effect of *lta4h* polymorphism on the outcomes of tuberculosis in humans; two of these studies included the efficacy of corticosteroids in their assessments, with contradicting findings. The initial study, including patients with TBM from Vietnam, found that treatment with corticosteroids was only beneficial in those with the hyperinflammatory TT genotype [96]; a later study, assessing the relation between *lta4h* genotype and TB-IRIS, found steroids were effective in treating TB-IRIS across all genotypes [99].

There are concerns regarding the use of corticosteroids as adjuvant treatment, especially in HIV co-infected patients: excess of Kaposi's sarcoma and other malignancies [88, 100]; a transient increase in HIV viral load [90]; worsening of underlying hypertension, fluid retention and hyperglycaemia [90]; and more reactivation of herpes zoster [101] have all been described in association with adjuvant corticosteroid use in HIV-associated tuberculosis, albeit predominantly in patients not on ART. Contrasting these findings, one study demonstrated fewer severe adverse drug reactions in patients receiving dexamethasone [93]; in particular, eight cases of severe drug-induced hepatitis occurred in the placebo group and none in the dexamethasone group.

The PredART trial

Study design, setting and participants

The PredART trial was a proof of concept, phase III, randomized, double blind, placebo-controlled trial of prednisone to assess its efficacy and safety in preventing paradoxical TB-IRIS in high-risk patients starting ART. Participants were recruited from four different tuberculosis clinics in Khayelitsha, a township 20 km from Cape Town's center with an estimated 500,000 inhabitants. At the time of recruitment, an estimated 16% of its population was HIV infected [102]; tuberculosis case notification was 917/100,000 per year and TB/HIV co-infection was 60% (City of Cape Town, 2015). Inclusion and exclusion criteria are shown in table 1.1. Informed consent was obtained for screening and again for enrolment for each participant. The protocol was approved by University of Cape Town Human Research Ethics Committee (HREC 136/2013), Institute of Tropical Medicine (ITM) Institutional Review Board (882/13), and the Antwerp University Hospital Ethical Committee (13/20/224).

TABLE 1.1 – Inclusion and exclusion criteria of the PredART trial

Inclusion criteria	Exclusion criteria
HIV-infected	Kaposi's sarcoma
CD4 count ≤ 100 cells /µl (in the past 3 months)	Pregnant
ART-naïve	< 18 years old
Confirmed diagnosis of TB or strong clinical and radiological evidence of TB with symptomatic response to TB treatment	TB meningitis or tuberculoma at TB diagnosis
	Clinical syndrome of pericardial TB at TB diagnosis
	Rifampicin-resistant TB
On TB treatment for less than 30 days prior to study entry	Uncontrolled diabetes mellitus
Eligible for ART and patient consents to start ART within 30 days of starting TB treatment	On corticosteroids for another indication or on any other immunosuppressive medication within the past 7 days
Written informed consent	The following abnormal laboratory values: Alanine aminotransferase > 200 IU/l Absolute neutrophil count < 500/mm^3
	Not on standard intensive phase TB treatment
	Poor clinical response to TB treatment prior to ART as judged by the clinical investigators
	Hepatitis B surface antigen positive

ART = antiretroviral therapy; TB = tuberculosis

Study drug and treatment of tuberculosis and HIV

Participants were randomized 1:1 to receive oral prednisone 40 mg daily for 14 days (started within 48 hours of initiating ART), followed by 20 mg daily for 14 days, or identical placebo. Tuberculosis was treated at participant's local clinic following the South African Department of Health guidelines [103] and consisted of weight-based daily doses of isoniazid (INH), rifampicin (RIF), pyrazinamide (PZA) and ethambutol (EMB) for 2 months,

followed by INH and RIF for another 4 months. ART was provided according to the South African Department of Health guidelines [104], the majority of participants receiving first line ART consisting of tenofovir (TDF) 300 mg, emtricitabine (FTC) 200 mg and efavirenz (EFV) 600 mg daily.

Trial procedures

Study visits occurred at screening, enrolment, week 0 (the day the participant starts study drug and ART), week 1, week 2, week 4, week 8, and week 12. Assessments done at each visit are summarized in Table 1.2. If the attending clinician suspected TB-IRIS, laboratory investigations, including a bacterial blood culture, and a chest radiograph were performed. Outside the scheduled visits, participants could attend for unscheduled visits, if they experienced symptomatic deterioration or if deemed necessary by the attending clinician.

TABLE 1.2 - Schedule of events

Study visit	**Scr**	**Enr**	**Wk 0**	**Wk 1**	**Wk 2**	**Wk 4**	**Wk 8**	**Wk 12**	**UV**
ART day			0	7 ± 4	14 ± 4	28 ± 4	56 ± 4	84 ± 7	Not specified
Document HIV status	x								
Screening ICF	x								
Enrolment ICF		x							
Study drug dispensed			x		x				
Symptoms[a]	x	x	x	x	x	x	x	x	x
Karnofsky score	x		x	x	x	x	x	x	x
Pill count[b]				x	x	x	x	x	x
HR-QoL assessments			x			x		x	
Examination	x	(x)	x	x	x	x	x	x	x
Laboratory investigations[c]	x	(x)	x	x	x	x		x	(x)
CD4 count, HIV viral load	x							x	
Serum HBsAg	x								
Serum CrAg	x								
Urinary pregnancy test	x	(x)	(x)	(x)	(x)	(x)	(x)	(x)	(x)
Storage bloods and immunology assays			x		x	x		x	If IRIS suspected
Storage urine			x					x	If IRIS suspected
Chest radiograph	(x)		x						If IRIS suspected
Sputum Xpert MTB/RIF[d], TB culture and DST	x					x		x	
Initiate ART			x						

Scr = screening visit; Enr = enrolment visit; UV = unscheduled visit; ART = antiretroviral therapy; ICF = informed consent form; HR-QoL = health-related quality of life; HBsAg= hepatitis B surface antigen; CrAg = cryptococcal antigen; TB = tuberculosis; DST = drug sensitivity testing; x = performed; (x) = if clinically indicated [a] This includes all symptoms, and specific screening for adverse events and TB-IRIS; [b] ART and study drug during week 1-4, ART thereafter; [c] Full blood count with leucocyte differentiation, sodium, potassium, creatinine, glucose, alanine aminotransferase, alkaline phosphatase, C-reactive protein; [d] Cepheid, Sunnyvale, CA, USA

For participants with ongoing TB-IRIS at week 12, follow-up was extended in order to ascertain the end date of TB-IRIS. During the course of this trial, results of the Investigation of the Management of Pericarditis (IMPI) trial [88] became available, showing an increased incidence of cancer in patients with HIV-related tuberculosis pericarditis prescribed prednisolone, compared to those prescribed placebo. On the basis of these results, we added one visit at week 28 and a telephonic follow-up at one year to monitor for HIV-related cancers. All other analyses remained restricted to data obtained over 12 weeks. When TB-IRIS was suspected, investigations mentioned above were performed with the main aim to exclude alternative causes of clinical deterioration. Adherence to ART, tuberculosis treatment and trial medication was also assessed using pill counts. If TB-IRIS was diagnosed, the study drug could be stopped and open label prednisone started, at a starting dose of 1.5 mg/kg/day, which was weaned over 4 weeks or longer depending on clinical response. The decision to treat with open label prednisone was made by the trial doctor.

Endpoints

The primary endpoint was the development of paradoxical TB-IRIS within 12 weeks of starting ART, defined using the International Network for the Study of HIV-associated IRIS (INSHI) consensus case definition [27]. This was adjudicated by a committee of three clinical experts not active at the clinical site who were given access to all clinical, laboratory data, and digital chest radiographs (CXR) and reports of other radiographic studies. They also

TABLE 1.3 – Secondary study endpoints

Secondary efficacy endpoints	Secondary safety and tolerability endpoints
Time to TB-IRIS event from start of ART	Corticosteroid-associated adverse events[a]
Severity of TB-IRIS events (defined by need for hospitalization, neurological involvement and C-reactive protein)	Laboratory safety data: glucose, full blood count and electrolytes
Duration of TB-IRIS events	Other infections and malignancies
Mortality attributed to TB and TB-IRIS	All grade 1, 2, 3, and 4 adverse events[b]
All-cause mortality	
Composite endpoint of death, hospitalization, or hepatotoxicity	
Other (non-TB) IRIS events	
Health-related quality of life assessments[c]	
Adverse events and severe adverse events ascribed to TB treatment or ART	
Discontinuation of ART or TB treatment for > 5 days due to adverse events	
Number of hospitalizations and total days hospitalized	

ART = antiretroviral therapy; TB = tuberculosis [a] These are pre-defined as hypertension, hyperglycaemia, hypomania/mania, depression, acne, epigastric pain, upper gastro-intestinal bleeding, Cushingoid features, new edema, and avascular bone necrosis. [b] Using the DAIDS AE grading table [105] [c] Using the Patient Reported Outcomes Quality of Life-HIV (PROQOL-HIV) [106], an adaptation of the HIV-symptom index by Justice [107], and the EQ-5D-3L [www.euroquol.org]

accessed TB-IRIS narrative summaries that were written by the trial doctor for any clinical deterioration after initiation of ART. After completion of the trial, committee members independently assessed all available information from each participant who experienced clinical deterioration after initiation of ART. Cases where there was disagreement, were resolved by consensus between the members.
Secondary endpoints are listed in table 1.3.

Statistical analysis

The primary end point and the secondary efficacy end points were analyzed using the intention- to-treat approach; secondary safety analyses using the all patients treated approach. The analysis of the primary end point was performed with the use of the chi-square test. Secondary end points were analyzed with the use of chi-square, Fisher's exact, or Wilcoxon rank-sum tests, as appropriate. Time to event analysis was used to compare time from the start of ART to TB-IRIS between study arms.

Main study findings

Participants

Between August 2013 and February 2016, 321 participants were screened and 240 randomized to receive either prophylactic prednisone or placebo (Figure 1.2). Two participants never started ART.

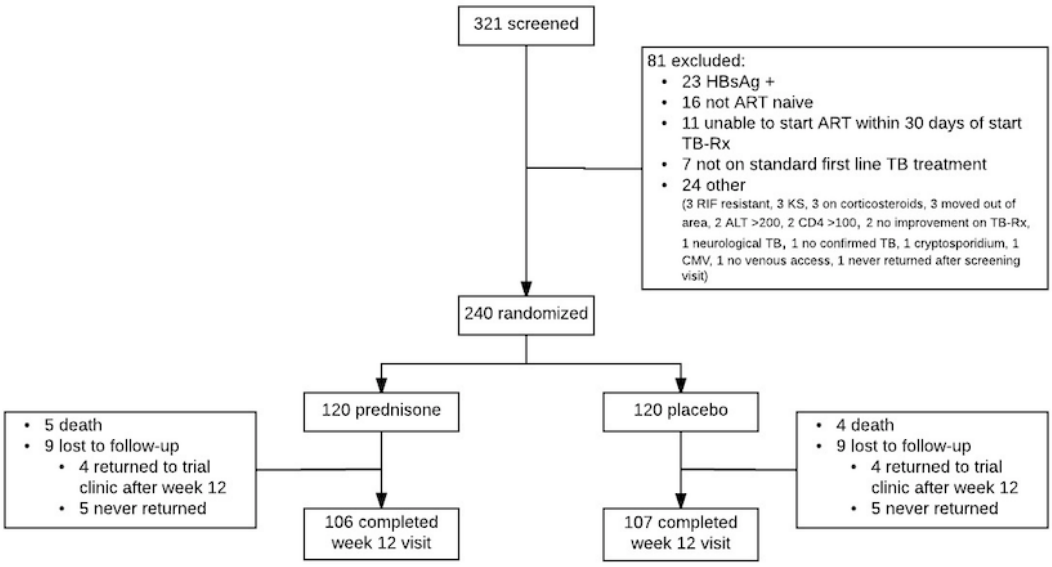


FIGURE 1.2 – Flow of participants in the PredART trial

Figure adapted from [43].

Baseline characteristics are summarized in Table 1.4. The median age of participants was 36 (IQR 30 - 42), 60% were men, and 73% had microbiologically confirmed tuberculosis; the median CD4 count was 49 cells / µl (IQR 24 - 86), and the median HIV viral load was 5.5 log10 copies per milliliter (IQR 5.2 - 5.9). Patients had received antituberculosis treatment for a median of 17 days before starting ART.

TABLE 1.4 – Baseline characteristics

	Prednisone arm (n=120)	**Placebo arm (n=120)**
Age (years)	36 (31-42)	36 (29-42)
Male sex	71 (59.2%)	73 (60.8%)
Body mass index (kg/m2)	21 (19-24)	21 (19-24)
CD4 count (cells/µl)	51 (27-84)	49 (23-88)
HIV RNA (log10 copies/ml)	5.5 (5.2-5.9)	5.6 (5.2-5.9)
Microbiologically confirmed TB[a]	86 (71.7%)	89 (74.2%)
Hemoglobin (g/dl)	9.7 (8.8-11.1)	9.8 (8.5-10.9)
White cell count (x 109 cells/l)	3.7 (2.9-5.1)	3.4 (2.6-5.0)
Neutrophil count (x 109 cells/l)	2.3 (1.5-3.1)	2.0 (1.4-2.9)
Platelet count (x 109 cells/l)	311 (259-413)	300 (226-396)
Sodium (mmol/l)	136 (134-139)	137 (135-139)
Creatinine (µmol/l)	57 (50-66)	59 (50-70)
Total bilirubin (µmol/l)	6 (4-7)	6 (4-8)
Alanine aminotransferase (IU/l)	26 (18-38)	28 (20-40)
Alkaline phosphatase (IU/l)	113 (87-149)	115 (91-163)
C-reactive protein (mg/l)	10.9 (4.0-30.1)	10.7 (4.6-29.9)
Karnofsky performance score	90 (80-90)	90 (80-90)
Duration TB treatment to ART (days)	16 (15-22)	17 (15-21)

Data are shown as medians (interquartile range) or number (percentage). ART = antiretroviral therapy; TB = tuberculosis [a] Microbiologically confirmed included Mycobacterium tuberculosis detected on culture or Xpert MTB/RIF assay, or positive acid fast-bacilli on microscopy. Table adapted from [43].

Primary and secondary end points

In the placebo arm 56/120 (46.7%) were diagnosed with paradoxical TB-IRIS, compared with 39/120 (32.5%) in the prednisone arm (p=0.03; RR 0.70; 95% CI 0.51 - 0.96) (Figure 1.3). The absolute difference in TB-IRIS incidence was 14.2% (95% CI 1.9 - 26.4).

The hazard of TB-IRIS was lower in the prednisone arm (hazard ratio 0.61; 95% CI 0.41 - 0.92) (Figure 2b). The median time to TB-IRIS symptom onset among the 95 participants diagnosed with TB-IRIS was similar by arm: 8 days in the placebo arm (IQR 4-12) and 10 days in the prednisone arm (IQR 5-13).

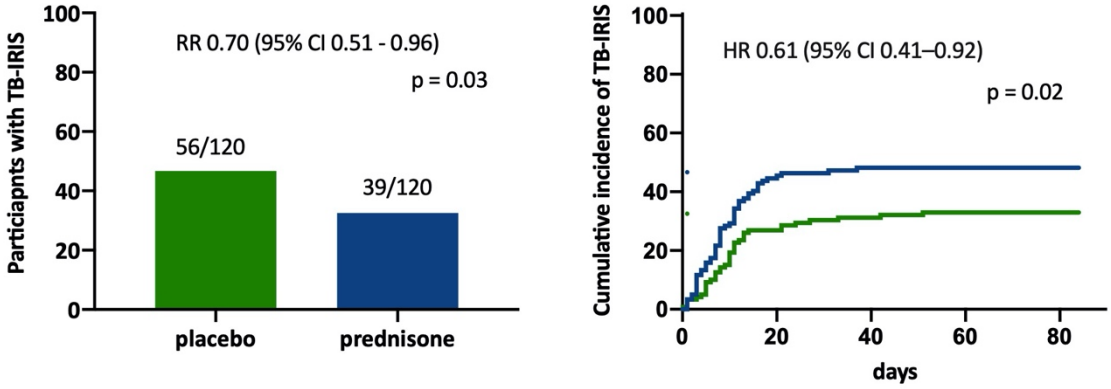


FIGURE 1.3 Cumulative incidence of TB-IRIS

Left: number of participants with TB-IRIS at week 12. Right: cumulative incidence of TB-IRIS over 84 days. Day 0 is the day antiretroviral therapy was started. RR = relative risk; HR = hazard ratio; CI = confidence interval. Figure adapted from [43]

In the prednisone arm, fewer participants fulfilled at least one major INSHI criterion (RR 0.57; 95% CI 0.37 - 0.87). Fewer participants were treated for TB-IRIS with open-label corticosteroids in the prednisone arm (RR 0.47; 95% CI 0.27 - 0.81). When restricted to the 95 participants diagnosed with TB-IRIS, 34/56 in the placebo arm (60.7%) and 16/39 in the prednisone arm (41.0%) were treated with open-label corticosteroids (RR=0.68; 95% CI 0.44 - 1.04, p=0.09). There was no statistically significant difference in any of the other secondary endpoints.

Lung function during and after tuberculosis

Tuberculosis is a risk factor for lung function impairment. Odds ratios for abnormal spirometry outcomes in patients with a history of tuberculosis range from 2.13 (95% CI 1.42 - 3.91) to 3.81 (95% CI 1.42 - 10.20) compared to those without such a history [108-111]. Both obstructive and restrictive impairment occur. The host immune response is thought to play an important role in the development of lung tissue damage during active tuberculosis leading to lung function impairment. Key mediators are matrix metalloproteinases, pro-inflammatory cytokines, eicosanoids and neutrophils. These immune mechanisms of parenchymal lung damage are reviewed in chapter 4 of this thesis, including the possibility of using host-directed therapies to intervene in these processes.

Studies evaluating lung function in patients during and after tuberculosis treatment find abnormal lung function in 45-87% [112-115], with even higher percentages in multi-drug resistant tuberculosis [116, 117]. With an estimated 10 million people falling ill with tuberculosis in 2018 [11], tuberculosis could well be one of the most important causes of chronic lung disease worldwide. However, little research has been done into post-

tuberculosis lung disease. Lung function data in patients with HIV-associated tuberculosis are even more scarce. HIV in itself is a risk factor for - mainly obstructive - lung function impairment [118-120]. At the same time, several factors suggest that tuberculosis associated lung damage might be less in patients co-infected with HIV: patients with HIV-associated tuberculosis and low CD4 counts (CD4 < 200/µl) often present with atypical chest radiograph (CXR) findings, or even normal CXRs [121]. Moreover, several of the inflammatory mediators involved in lung damage associated with tuberculosis are attenuated by HIV co-infection [122, 123]. By contrast, inflammation and inflammatory mediators are increased during TB-IRIS, suggesting that TB-IRIS could result in more lung function impairment. Evidence from available studies for both the effect of HIV co-infection and TB-IRIS on lung function is conflicting. One study assessing post-tuberculosis lung disease in patients with and without HIV co-infection found less pulmonary impairment in HIV positive patients [114], but other studies did not find this association between HIV status and spirometry outcomes [115, 124, 125]. As for TB-IRIS, recent studies suggest TB-IRIS may cause lung function impairment [79, 126, 127], but in these studies the number of patients developing TB-IRIS was small. In the first study [79], lung function tests were performed in 14 patients 2 years after completion of treatment for HIV-associated tuberculosis. In the three patients that had developed TB-IRIS, lower FEV1 values (FEV1 percentage of predicted 65% vs 79%) as well as a lower FEV1/FVC ratio (FEV1/FVC percentage of predicted 75% vs 100%) were found compared to the non-IRIS controls. In the second study [126], 63 patients treated for HIV-associated tuberculosis underwent longitudinal lung function tests, of whom 23 also underwent 18[F] fluorodeoxyglucose (FDG) positron emission tomography – computed tomography (PET-CT) scans. More inflammation on the PET-CT scan associated with worse lung function at baseline (start of ART). An increase in inflammation over time was seen in the 12 patients that showed a decrease in lung function between baseline and week 4. Moreover, the authors describe an association between increased inflammation over time and lower baseline CD4 count, higher baseline HIV viral load and shorter time interval between the start of anti-tuberculosis therapy and ART, although only the latter was statistically significant. They reason increased inflammation may be due to undiagnosed TB-IRIS (only 3 patients developed clinical TB-IRIS), which may then be associated with impaired lung function. In a third study [127] by the same authors using the same patient cohort, longitudinal lung function tests were performed in 101 patients, six of whom developed TB-IRIS. The authors focus on the 50 patients with a significant decline in lung function over time (drop in FEV1 > 100ml) and describe a 'substantial virologic decreases and CD4 increases on ART' (actual data not available). Reasoning along the same lines as in their previous paper, they suggest a relation between decrease in lung function and TB-IRIS and state that otherwise unexplained drops in pulmonary function on ART warrant consideration as an additional criterion for a TB-IRIS diagnosis. FEV1 at 48 weeks was similar for patients with and without initial lung function decline.

Objectives and outline

The main objective of this thesis is to assess the effect of prednisone on immunopathological mediators and pulmonary function in HIV-associated tuberculosis and TB-IRIS (chapters 3 and 5). As a secondary objective I evaluated and investigated ways to validate and enhance the International Network for the Study of HIV-associated IRIS (INSHI) consensus case definition for paradoxical TB-IRIS [27] (chapter 2).

The diagnosis of paradoxical TB-IRIS relies on characteristic clinical features that have been synthesized in the International Network for the Study of HIV-associated IRIS (INSHI) consensus case definition. There is no confirmatory laboratory test. In **chapter two**, I report a study in which I applied latent class analysis to model a potential gold standard case definition for TB-IRIS, using data from 217 participants in the PredART trial. I used the model-predicted probability of TB-IRIS for each participant to assess the performance of the INSHI case definition and compare its diagnostic accuracy with several adapted case definitions. I did the statistical analyses during my stay in Antwerp with the oversight of biostatistician Dr. Jozefien Buyze and the advice of Dr. Joris Menten. In this chapter, I describe the diagnostic accuracy of the INSHI case definition and several proposed amended case definitions. The contents of this chapter have been published as: Stek C, Buyze J, Menten J, Schutz C, Thienemann F, Blumenthal L, Maartens G, Boyles T, Wilkinson RJ, Meintjes G, Lynen L. *Diagnostic accuracy of the INSHI consensus case definition for the diagnosis of paradoxical tuberculosis-IRIS*. J Acquir Immune Defic Syndr. 2020 Dec 17. I led the writing of the manuscript as first author.

In **chapter three**, I report a study in which I investigated the associations between cytokine/chemokine profiles, LTA4H genotype, development of TB-IRIS and use of prophylactic prednisone. I used stored plasma from 183 PredART participants, taken at baseline (start of antiretroviral therapy (ART) and 2 weeks after the start of ART to measure levels of 31 cytokines and chemokines. I determined *lta4h* genotype of 213 PredART participants using DNA extracted from stored blood. I have extracted the DNA from the stored samples and performed the chemo- and cytokine assays under the supervision of Dr. Muki Shey. Khuthala Mnika conducted the genotyping in the laboratory. I describe the association between cytokine profiles and TB-IRIS at baseline and week 2, and the effect of prednisone prophylaxis on cytokine levels. I also describe the association between TB-IRIS, efficacy of prednisone and LTA4H genotype. I have written the protocol for this substudy, analyzed the data, and written the results.

Chapter four provides a comprehensive review of the mechanisms of lung damage in tuberculosis and potential strategies to prevent it. I first review the literature that has

characterised lung damage in human tuberculosis. Next, I give an overview of the different immune pathways involved in lung damage in tuberculosis, once again focusing on findings in human tuberculosis and highlighting some of the challenges related to research in this area. Based on these findings, I discuss the potential of certain registered medications to serve as host-directed therapies to reduce lung damage and review the available evidence for their use. This chapter serves as an introduction to chapter five, in which I describe a lung function substudy conducted within the PredART trial. The contents of chapter four have been published; I conducted the literature review for this publication and led the writing of the manuscript as first author (Stek, C., Allwood, B., Walker, N. F., Wilkinson, R. J., Lynen, L., Meintjes, G., *The Immune Mechanisms of Lung Parenchymal Damage in Tuberculosis and the Role of Host-Directed Therapy*. Front Microbiol, 2018. 9: p. 2603).

Chapter five describes a study of lung function impairment during and after completion of treatment for HIV-associated tuberculosis. I determined the prevalence of lung function abnormalities in patients with HIV-associated tuberculosis and CD4 counts < 100 cells/µl and assessed the effect of prophylactic prednisone and the development of paradoxical TB-IRIS on lung function impairment. I have developed the protocol for this substudy. Together with research worker Nobom Masimini, I performed spirometry and six-minute walk test at baseline (week 0), week 4, 12, and 28 in 153 participants of the PredART trial. I scored chest radiographs performed at baseline and week 28 together with Dr. Elsa du Bruyn under the supervision of Dr. Brian Allwood. Statistical analysis of the data was done in close collaboration with Dr. Jozefien Buyze. In this chapter, I present the findings of this PredART substudy, published in Stek C, Allwood B, Du Bruyn E, Buyze J, Schutz C, Thienemann F, Lombard A, Wilkinson RJ, Meintjes G, Lynen L., *The effect of HIV-associated tuberculosis, tuberculosis-IRIS and prednisone on lung function,* Eur Respir J. 2020 Mar 12;55(3).

In **chapter six** I summarize the findings of the studies, draw broad conclusions from across the studies presented and provide recommendations for future research in these areas.

All chapters of this thesis are substudies of the PredART trial [43]. I played a central role in the recruitment and follow-up of the participants on this trial, as well as in the collection, management, cleaning, and analysis of the data.

The PredART trial was approved by the University of Cape Town Human Research Ethics Committee (HREC ref nr 136/2013), Institute of Tropical Medicine Institutional Review Board (IRB nr 882/13), and the Antwerp University Hospital Ethics Committee (UA ref nr 13/02/224). The substudies have been approved by the same ethical committees that approved the main trial (for the laboratory study: HREC ref nr 136/2013; IRB nr 1246/18; UA ref nr 18/40/425; for the lung function study HREC ref nr 925/2014; IRB nr 974/14; UA ref nr 14/46/471).

Diagnostic accuracy of the INSHI consensus case definition for the diagnosis of paradoxical tuberculosis-IRIS

Introduction

Paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) is an immunopathological reaction resulting in new or recurrent TB signs and symptoms, shortly after starting antiretroviral therapy (ART). It complicates treatment of HIV-associated tuberculosis (TB) in approximately 18% of cases, with rates more than 50% in high risk groups, causing significant morbidity [18, 80]. The diagnosis of TB-IRIS is largely based on its characteristic clinical presentation; there is no confirmatory laboratory test. The International Network for the Study of HIV-associated IRIS (INSHI) consensus case definition is used to diagnose TB-IRIS [27]. It requires a diagnosis of TB with an initial positive response to treatment, characteristic clinical features (such as new or enlarging lymphadenopathy, constitutional, respiratory or abdominal symptoms, or new or worsening radiological features), and exclusion of alternative explanations for clinical deterioration – see Panel 2.1. It is designed for use by both clinicians and researchers in a variety of settings and has been validated in several studies [31-33]. However, in the absence of a gold standard, validation was always performed against diagnostic assignment based upon expert opinion. Having a gold standard for TB-IRIS would allow for a more objective validation of the INSHI case definition and assessment of the ability of new variables to improve its diagnostic accuracy.

Latent class analysis (LCA) is a modelling technique that can identify unobserved groups, or classes, in a population. It has been used and shown utility in several diseases characterized by the lack of a gold standard, such as latent TB or sepsis [128-132]. LCA divides the population in two groups: those who have the disease, and those who do not have the disease. In the absence of a diagnostic standard for the disease, the disease itself cannot be measured directly. Instead, many clinical signs and symptoms exist, that are each in themselves imperfect predictors of the disease. These signs and symptoms cluster in different patterns in different individuals. The observed frequencies of these patterns allow for the construction of models estimating the prevalence of the disease and the sensitivity and specificity of each sign or symptom, or combinations thereof.

(A) Antecedent requirements
Both of the two following requirements must be met:

- Diagnosis of tuberculosis
- Initial response to tuberculosis treatment

(B) Clinical criteria
The onset of TB-associated IRIS manifestations should be within 3 months of ART (re)initiation or regimen change because of treatment failure. Of the following, at least one major or two minor criteria are required:
Major criteria

- New or enlarging lymph nodes, cold abscesses, or other focal tissue involvement
- New or worsening radiological features of tuberculosis
- New or worsening central nervous system tuberculosis
- New or worsening serositis

Minor criteria

- New or worsening constitutional symptoms such as fever, night sweats or weight loss
- New or worsening respiratory symptoms such as cough, dyspnea, or stridor
- New or worsening abdominal pain accompanied by peritonitis, hepatomegaly, splenomegaly or abdominal adenopathy

(C) Alternative explanations for clinical deterioration must be excluded if possible

- Failure of tuberculosis treatment because of tuberculosis drug resistance
- Poor adherence to tuberculosis treatment
- Another opportunistic infection or neoplasm
- Drug toxicity or reaction

PANEL 2.1: INSHI case definition for paradoxical tuberculosis associated IRIS [27]

We used LCA to generate a surrogate gold standard for TB-IRIS. Next, we used the LCA-predicted probability of TB-IRIS for each participant to assess the performance of the INSHI case definition and compare its diagnostic accuracy with several adapted case definitions. We assessed the ability of additional variables to improve the diagnostic accuracy of the case definition. Finally, we assessed the ability of C-reactive protein (CRP) as a rule-out test for TB-IRIS.

Methods

Main trial and setting

We performed a retrospective analysis of data collected in the PredART trial [43]. This randomized, double-blind, placebo-controlled trial showed that a 28-day course of prophylactic prednisone in adult patients identified as being at high risk (time between initiation of antituberculosis treatment and ART < 30 days; CD4 count ≤100 cells/µl) for paradoxical TB-IRIS reduced the incidence of paradoxical TB-IRIS, without an excess of

adverse events. Between August 2013 and February 2016, the trial enrolled 240 participants from Khayelitsha, Cape Town, South Africa. Their median age was 36, 60% were men, and their median CD4 count was 49 cells/µl. The primary endpoint of the trial was the development of TB-IRIS, which was adjudicated by a committee of three clinical experts not active at the clinical site who were given access to all clinical, laboratory data, and digital chest radiographs (CXR) and reports of other radiographic studies. They also accessed TB-IRIS narrative summaries that were written by the trial doctor for any clinical deterioration after initiation of ART. After completion of the trial, committee members independently assessed all available information from each participant who experienced clinical deterioration after initiation of ART. The INSHI consensus case definition was used to diagnose TB-IRIS (INSHI TB-IRIS). In addition, committee members were given the option to indicate when they considered that a participant developed TB-IRIS, even though not fulfilling the INSHI case definition (paradoxical TB-IRIS not fulfilling the INSHI criteria); these participants were included in the analyses and defined as not having TB-IRIS according to INSHI criteria. Cases where there was disagreement, were resolved by consensus between the members.

Selection of variables

As variables for our latent class model we included the following: baseline participant characteristics (sex, age, details about current and previous TB episodes); INSHI major criteria; all individual symptoms of the INSHI minor criteria; any additionally documented signs and symptoms provided they occurred in more than 10 (4%) participants and could feasibly be related to TB-IRIS; baseline (immediately prior to starting ART) and follow-up laboratory variables (hemoglobin, leucocytes, creatinine clearance, alanine transferase, alkaline phosphatase, CRP, CD4 count and HIV viral load); signs and symptoms that were identified as possibly related to TB-IRIS during the TB-IRIS adjudication process; and baseline urine lipoarabinomannan (LAM), determined retrospectively on stored urine using Alere Determine lateral flow assay.

Data for variables included were restricted to the first four weeks after ART initiation (95% of the TB-IRIS cases in our cohort occurred within four weeks, with a median time between ART initiation and TB-IRIS of 8 (IQR 5-13) days).

In LCA as we applied it, variables need to be binary: a sign or symptom was either present or absent in the first four weeks. Therefore, continuous variables were transformed into binary ones by plotting them in a receiver operating characteristics (ROC) curve and - after excluding variables with ROC area under the curve < 0.55 - selecting a cut-off that obtained the maximum of the sum of the computed sensitivities and specificities. For these variables to be positive, the highest (or, for hemoglobin (Hb), the lowest) value of that variable measured anytime during the first four weeks needed to be higher (lower for Hb) than the cut-off.

Given the relatively small number of participants in the study, only a limited number of variables could be included in the LCA model to avoid overfitting the model to the data.

To select which variables to include in the latent class model, we first calculated the unadjusted odds ratio (OR) for each individual variable and the consensus decision of the adjudication committee for INSHI TB-IRIS, using Stata version 14.2. These are listed in Table 2.1. We combined the abdominal symptoms associated with TB-IRIS into one new variable, that comprised 'abdominal pain with either hepatomegaly, splenomegaly, or abdominal lymph nodes, or abdominal tenderness on clinical examination without other explanation'. We combined cough, dyspnea and chest pain into the variable 'respiratory symptoms'. Both new variables did not affect the selection of any other variable for inclusion in the latent class model.
We assessed different unadjusted ORs as cut-off for variable inclusion in the multivariable model: OR > 2.0 or < 0.5 (2-model), OR > 3.0 or < 0.3 (3-model), and OR > 5.0 or < 0.2 (5-model). We chose the 3-model, because it was the most parsimonious.
We did not include urine LAM in the initial latent class model, because of a higher number of participants with missing data (n = 35). Rather, we used a model containing urine LAM as an alternative model to compare findings with our final model.
Next, we combined variables selected based on their unadjusted OR in a backward multiple logistic regression model. Those variables with a p-value < 0.1 were retained and entered in the latent class model, together with the INSHI major criteria. The LCA model was limited to a maximum of ten variables to avoid too many variable combinations with no or very few observations. This limits the number of observations to nine per variable, in line with the recommendation to limit the number of variables in a logistic regression model to one per ten cases [133].

Latent class model

We performed LCA using LEM software [J. Vermunt, Tilburg, The Netherlands, 1993; https://jeroenvermunt.nl]. We assumed the presence of two latent classes, corresponding to TB-IRIS and no TB-IRIS. We initially fitted models applying the conditional independence assumption. Goodness of fit tests – using the Chi-squared test - confirmed there was no requirement to include conditional dependencies between variables in the LCA model. We excluded 21 participants with missing data on any of the selected variables as LEM does not allow missing values. Baseline variables for these participants were comparable to those of the included participants (data not shown).

Diagnostic accuracy of various case definitions and CRP

After running the latent class model, we used the LCA-predicted probability of TB-IRIS for each participant to first calculate the positive and negative predictive values for each sign or symptom initially considered for LCA, followed by their sensitivity and specificity, again using Stata version 14.2. We combined the individual signs and symptoms to match the INSHI consensus case definition or form several adapted case definitions. Doing so, we could assess the performance of the INSHI case definition and compare its diagnostic accuracy

with the adapted case definitions. We also assessed the diagnostic ability of CRP to rule-out TB-IRIS, using a range of lower cut-off values, aiming for a high sensitivity.

Results

Out of the 240 participants in the PredART trial, complete data for latent class modelling were available for 217 participants (Figure 2.1). Baseline variables for these 217 were comparable to those of all participants in the PredART trial (data not shown).

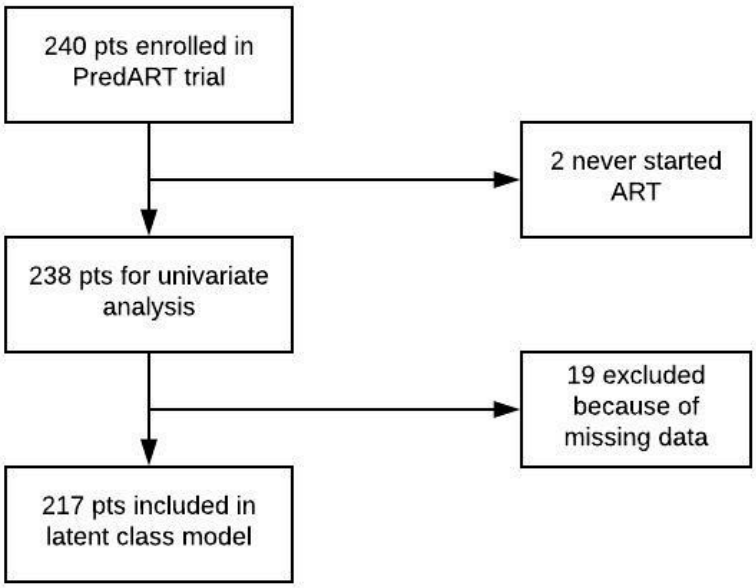


FIGURE 2.1 Flow of participants

Presentation of paradoxical TB-IRIS

Eighty-nine participants (41%) developed TB-IRIS according to the INSHI criteria. Twelve (6%) participants were adjudicated by the committee to have paradoxical TB-IRIS not fulfilling the INSHI criteria. Of the INSHI TB-IRIS cases, 66 (74%) fulfilled at least one INSHI major criterion, criteria 1 and 2 (new or enlarging lymph nodes or new or worsening chest CXR abnormalities) being the most frequent. None of the participants fulfilled INSHI major criterion 3 (neurologic features). All but one of the participants who developed TB-IRIS also fulfilled at least one minor criterion. Twenty-three participants (26%) had a diagnosis of TB-IRIS based on having two or more minor criteria without a major criterion. Of the participants without TB-IRIS, 26 (20%) fulfilled one minor criterion; this included all 12 participants assessed by the committee to have paradoxical TB-IRIS, but not fulfilling the INSHI criteria.

TABLE 2.1 Variables initially included in the selection for the latent class model, their unadjusted odds ratio for INHSI IRIS, and their occurrence in participants with and without TB-IRIS according to the INSHI consensus case definition.

	Unadjusted odds ratio for INSHI IRIS (n = 238)	INSHI-IRIS (n = 89)		No INSHI-IRIS (n = 128)	
Baseline variables					
Sex (M)	0.8	56	(63%)	76	(59%)
Age (< 40 y)	2.1	67	(75%)	76	(59%)
Previous tuberculosis	0.9	8	(9%)	14	(11%)
Microbiological evidence of tuberculosis	1.8	71	(80%)	89	(70%)
Time from start of tuberculosis treatment to start of antiretroviral therapy (< 14d)	0.9	85	(96%)	89	(70%)
Baseline CD4 count (< 50 cells/µl)	3.5	70	(79%)	62	(48%)
Baseline HIV viral load (< 150000 cp/ml)	1.0	12	(14%)	38	(30%)
Baseline urine LAM (≥ 1)	4.0	62	(73%)	45	(38%)
INSHI related variables					
Major criteria					
Enlarged lymph nodes	-	30	(34%)	0	(0%)
New/worsening radiological features	-	40	(45%)	0	(0%)
Central nervous system TB-IRIS[a]	-	0	(0%)	0	(0%)
New/worsening serositis	-	3	(3%)	0	(0%)
Minor criteria					
Weight loss[b]	4.0	45	(51%)	27	(21%)
Fever[c]	8.7	33	(37%)	8	(6%)
Night sweats	8.4	50	(56%)	14	(11%)
Anorexia	3.2	47	(53%)	32	(25%)
Weakness	3.8	30	(34%)	13	(10%)
Cough	3.9	32	(36%)	16	(13%)
Chest pain	6.4	24	(27%)	8	(6%)
Dyspnea	8.3	22	(25%)	4	(3%)
Abdominal pain with hepatomegaly, splenomegaly or enlarged abdominal lymph nodes	4.3	8	(9%)	3	(2%)

TABLE 2.1 - continued

Included other adverse events[d]					
Headache	3.8	22	(25%)	11	(9%)
Dizziness	0.5	6	(7%)	10	(13%)
Arthralgia	1.1	6	(7%)	8	(6%)
Back pain	0.6	4	(4%)	8	(6%)
Flank pain	1.3	5	(6%)	5	(4%)
Epigastric pain	1.5	5	(6%)	5	(4%)
Abdominal pain	4.5	20	(22%)	8	(6%)
Nausea	2.8	27	(30%)	17	(13%)
Vomiting	2.5	34	(38%)	25	(20%)
Diarrhea[e]	4.3	28	(31%)	9	(7%)
Herpes simplex infection	2.2	17	(19%)	13	(10%)
Pruritis	1.1	6	(7%)	7	(5%)
Paresthesia	0.9	7	(8%)	10	(8%)
Papular pruritic eruption	0.4	3	(3%)	11	(9%)
Variables raised during TB-IRIS adjudication					
Tachycardia (heart rate > 120/min)	4.9	62	(70%)	39	(30%)
Return of any (non-INSHI) initial tuberculosis symptom	2.5	21	(24%)	12	(9%)
Abdominal pain and tenderness without any other explanation	14.9	9	(10%)	1	(1%)
Raised C-reactive protein	5.7	55	(62%)	27	(21%)
Laboratory variables[f]					
Haemoglobin (< 10 g/dl)	2.0	52	(58%)	51	(39%)
Leucocytes (> 10 x 10^9 cells/l)	1.0	33	(37%)	11	(9%)
C-reactive protein (> 90 mg/l)	5.7	55	(62%)	27	(21%)

Unadjusted odds ratios were computed including all participant of the PredART trial who had taken at least one dose of ART. Comparison between participants with and without TB-IRIS was done in participants included in the latent class model. [a] None of the participants developed neurological TB-IRIS [b] Weight loss is defined as > 2.5% in 2 weeks or > 5% in 4 weeks [c] Fever is defined as temperature > 37.7 ^{0}C, following the DAIDS table for grading the severity of adverse events [105] that was current during the collection of data [d] The following adverse events were excluded from analysis because of an unlikely association with TB-IRIS: upper respiratory tract infection (including coryza), blocked nose, ear symptoms, tooth ache, heartburn, hemorrhoids, swollen feet, a patient history of feeling hot or cold, scabies, urinary tract infection, and low potassium. [e] This includes both diarrhea and 'more frequent than normal loose stools' [f] The following laboratory variables were excluded from analysis because the area under the curve in the receiver operating characteristic curve was < 0.55: creatinine clearance, alanine transferase, alkaline phosphatase, increase in CD4 count at week 12, decrease in HIV viral load at week 12

The most frequent symptoms in participants with INHSI TB-IRIS were respiratory symptoms (65%), night sweats (56%), loss of appetite (53%) and loss of weight (51%), followed by vomiting (38%), diarrhea or loose stool (31%) and weakness (34%). Fever (temp > 37.7 ^{0}C) was present in 37% of the participants with INSHI TB-IRIS, and tachycardia (heart rate > 120/min) in 70%. Sixty-two percent had a CRP > 90 mg/l (Table 2.1).

TABLE 2.2: LCA-predicted sensitivity and specificity of variables included in the final latent class model.

Variable	LCA predicted sensitivity	LCA predicted specificity
Respiratory symptoms	0.57	0.79
Night sweats	0.58	0.91
INSHI major 1 (new or enlarging lymph nodes)	0.28	0.96
INSHI major 2 (new or worsening CXR abnormalities)	0.38	0.96
INSHI major 4 (new or worsening serositis)	0.02	0.99
Maximum C-reactive protein > 90 mg/l	0.73	0.88
Maximum heart rate > 120 bpm	0.79	0.78
Maximum temperature > 37.7 ^{0}C	0.44	1.00
Nadir CD4 count < 50 cells/µl	0.81	0.54

Latent class model

The final latent class model included 9 variables: respiratory symptoms, night sweats, INSHI major criteria 1, 2, and 4 (new or enlarging lymph nodes, radiological abnormalities, and serositis respectively), maximum CRP > 90 mg/l, maximum heart rate > 120/min, maximum temperature > 37.7 ^{0}C, and pre-ART CD4 count < 50 cells/µl. The model showed a good fit to the data ($\chi 2 = 337$, $p = 1.0$). The model-estimated incidence of TB-IRIS was 43%. The predicted sensitivities and specificities of the variables included in the model are summarized in Table 2.2. Other models, eg. a model with the 5-model selected variables, a model including baseline urine LAM instead of maximum CRP, or a model including weight loss instead of fever showed similar results to the selected model (Table 2.3)

TABLE 2.3 LCA-predicted sensitivity and specificity of variables in alternative latent class models.

	Model with variables selected based on unadjusted OR > 5.0 or < 0.2 (5-model)		**Model replacing maximum CRP with baseline urine LAM**		**Model replacing fever with weight loss**	
Variable	LCA predicted sensitivity	LCA predicted specificity	LCA predicted sensitivity	LCA predicted specificity	LCA predicted sensitivity	LCA predicted specificity
Respiratory symptoms	0.59	0.84	0.60	0.83	0.56	0.81
Night sweats	0.61	0.98	0.59	0.96	0.54	0.92
INSHI major 1 (new or enlarging lymph nodes)	0.27	0.97	0.28	0.99	0.27	0.98
INSHI major 2 (new or worsening CXR abnormalities)	0.36	0.97	0.36	0.96	0.34	0.96
INSHI major 4 (new or worsening serositis)	0.02	0.99	0.02	1.00	0.03	1.00
Maximum C-reactive protein > 90 mg/l	0.66	0.86	-	-	0.71	0.92
Maximum heart rate > 120 bpm	-	-	0.76	0.77	0.76	0.79
Maximum temperature > 37.7 ^{0}C	0.41	1.00	0.42	0.99	-	-
Nadir CD4 count < 50 cells/µl	-	-	0.79	0.54	0.78	0.54
Baseline urine LAM (≥ 1)	-	-	0.76	0.67	-	-
Weight loss	-	-	-	-	0.58	0.89

Comparison of case definitions

Using the model-predicted probability of TB-IRIS for each participant, we found the INSHI consensus case definition had a sensitivity of 0.77 and a specificity of 0.86. We constructed several adapted case definitions, replacing one or two of the required INSHI minor criteria with one or more of the model-derived variables CRP (> 90 mg/l), heart rate (> 120/min) or fever (temperature > 37.7 ^{0}C). The adapted case definitions had sensitivities and specificities similar to the INSHI consensus case definition. A definition replacing all the minor criteria with objective measures (CRP elevation, fever, and/or tachycardia) showed better diagnostic accuracy, with a sensitivity of 0.89 and a specificity of 0.88. Performance of the INSHI case definition and the adapted case definitions to identify TB-IRIS is summarized in Table 2.4.

TABLE 2.4: Diagnostic accuracy of INSHI and adapted case definitions.

TB-IRIS definition	Sensitivity	Specificity	LR+	LR-
INSHI case definition: presence of at least • 1 INSHI major criterion *OR* • 2 INSHI minor criteria	0.77	0.86	5.50	0.27
CRP > 90 mg/l	0.73	0.88	6.08	0.31
Baseline urine LAM ≥ 1	0.76	0.67	2.30	0.36
Presence of at least 1 INSHI major criterion only	0.60	0.92	7.50	0.43
Presence of at least • 1 INSHI major criterion *OR* • 2 INSHI minors criteria *OR* • 1 INSHI minor criterion and CRP > 90 mg/l	0.85	0.78	3.86	0.19
Presence of at least • 1 INSHI major criterion *OR* • 1 INSHI minor criterion and CRP > 90 mg/l	0.86	0.83	5.06	0.17

Presence of at least
• 1 INSHI major criterion *OR*
• 1 INSHI minor criterion and one of the following:
  ○ CRP > 90 mg/l
  ○ heart rate > 120/min
  ○ temperature > 37.7^0C
	0.93	0.82	5.17	0.09

Presence of at least
• 1 INSHI major criterion *OR*
• two of the following:
  ○ CRP > 90 mg/l
  ○ heart rate > 120/min
  ○ temperature > 37.7^0C
	0.89	0.88	7.42	0.13

LR+ = positive likelihood ratio; LR- = negative likelihood ratio
Case definitions were constructed, and their sensitivity, specificity, and positive and negative likelihood ratios computed using the LCA-predicted probability of TB-IRIS for each participant.

CRP to rule out TB-IRIS

Using the model-predicted probability of TB-IRIS for each participant, we found CRP values of > 10 mg/ml to > 50 mg/ml all had a sensitivity above 0.9 and a negative likelihood ratio of ≤ 0.15 (indicating a moderate to large decrease in probability of having a CRP value lower than the cut-off when having TB-IRIS) (Table 2.5). The area under the ROC curve was 0.86. The association between CRP and likelihood of TB-IRIS is shown in Figure 2.1, showing the inverse likelihood ratio, which indicates how many times less likely each CRP value is associated with TB-IRIS. We repeated the analysis using an alternative model including baseline urine LAM instead of CRP to confirm our findings; this analysis showed similar results (data not shown).

TABLE 2.5: LCA-predicted sensitivity and negative likelihood ratio of different cut-off values of CRP

CRP value (mg/ml)	LCA predicted sensitivity	LCA predicted negative likelihood ratio
> 10	0.99	0.07
> 20	0.97	0.09
> 30	0.95	0.13
> 40	0.93	0.15
> 50	0.92	0.13
> 60	0.88	0.20
> 70	0.80	0.27
> 80	0.77	0.28
> 90	0.73	0.30
> 100	0.64	0.39

Discussion

Because of the lack of a gold standard, validation of the INSHI consensus case definition for paradoxical TB-IRIS has previously been performed against diagnostic assignment based upon expert opinion [31, 32] or another expert-opinion based case definition [33], showing sensitivities of 0.72-0.91 and specificities of 0.93-1.0. We applied LCA, using participant data from a prospective trial of a TB-IRIS prevention strategy, to provide a surrogate gold standard for TB-IRIS. Participants included in this trial were all co-infected with HIV and TB and starting ART. This surrogate gold standard enabled us to validate the INSHI consensus case definition and confirm its reasonable diagnostic accuracy found in the previous studies using a data-derived approach.

The INSHI consensus case definition consists of 3 components: a prerequisite of a diagnosis of TB with an initial positive response to treatment, clinical features summarized in a combination of major and minor criteria, and exclusion of other reasons for clinical deterioration. The first and the last component are largely unambiguous. However, many of the INSHI minor criteria have a subjective component and depend on patient reported symptoms. Replacing these with objective variables could make the definition more robust and improves uniformity, which is advantageous if the definition is to be used for endpoint definition in clinical trials. We found that amending the case definition by replacing the INSHI minor criteria with the more objective variables tachycardia, fever, and/or CRP elevation improved sensitivity without loss of specificity.

CRP in itself may have utility as a rule-out test for TB-IRIS. We assessed this diagnostic ability of CRP and found that a normal CRP (< 10 mg/l) can be used to rule out TB-IRIS. Because the majority of patients with HIV-associated TB will however have a CRP > 10 mg/l [134-136],

we also assessed higher CRP cut-offs. Figure 2.1 showed that especially a low CRP is useful in excluding TB-IRIS, but higher cut-off values of CRP still have added value in ruling-out TB-IRIS. A CRP value of 82.4 corresponds to the cut-off that is equally likely for patients with or without TB-IRIS.

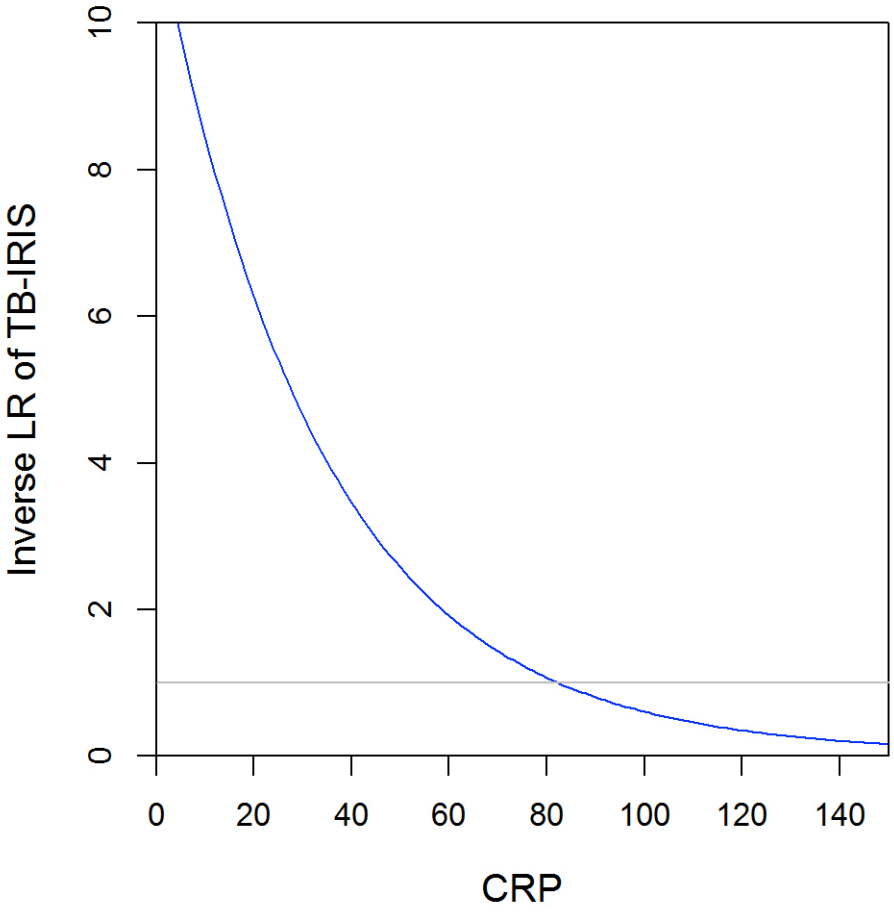


FIGURE 2.1 The association between CRP and likelihood of TB-IRIS

CRP (mg/l) is plotted against the inverse likelihood ratio of TB-IRIS, as predicted by the latent class model. The inverse likelihood ratio indicates how many times less likely each CRP value is associated with TB-IRIS.

Our study has several limitations: first, a relatively small study cohort and inclusion of a large number of possible variables in the latent class model resulted in the need to pre-select variables based on their univariate association with TB-IRIS so as not to exceed 10 variables in the model. Consequently, some important variables may have been excluded. However, we explored different selection criteria which resulted in similar results giving assurance of the robustness of the model. Second, there were no participants with a temperature above 37.7 ^{0}C among those who were classified as not having TB-IRIS according to the latent class models. Leaving temperature out of the model did not affect

this finding. Looking at the raw data, however, only four of the 41 participants with a temperature above 37.7 ^{0}C did not have TB-IRIS, according to either the INSHI case definition or the adjudication committee; all four had a more likely alternate diagnosis (gastro-intestinal infection, drug resistant TB, drug rash and drug-induced liver injury). Third, in the PredART trial CXRs were only repeated on ART when suspected TB-IRIS or other clinical deterioration prompted the clinician to request a CXR. As a consequence, we do not have documented normal CXRs for all participants who did not develop TB-IRIS, and therefore cannot say with certainty that none of the participants in this group had new or worsening CXR features. However, in the absence of other symptoms one can question its clinical relevance. Fourth, this was a restricted patient population, purposefully selected for its high risk of TB-IRIS: only patients with a CD4 count $\leq$ 100 cells/µl and antituberculosis treatment for $\leq$ 30 days before starting ART were included in the trial. It could be our model performs differently in a population with a lower incidence of TB-IRIS. Moreover, our findings only apply to patients with HIV-associated TB starting ART and not to paradoxical reactions that may occur in HIV-negative patients undergoing immune reconstitution for other reasons. Fifth, the method we used allows for only the estimation of the diagnostic indicators of variables included in the model as well as other variables. Calculation of the uncertainty and resulting confidence intervals of these estimates is however complex and is hampered by the inherent multiplicity due to variable selection in LCA model building. For this reason, we did not estimate confidence intervals for diagnostic accuracy measures in this analysis. Consequently, these estimates should be seen as exploratory and will need validation in an independent dataset to correctly estimate the bias and uncertainty in these estimates.

In conclusion, we found that the INSHI case definition identifies TB-IRIS with reasonable accuracy. Amending the case definition by replacing the INSHI minor criteria with the objective variables tachycardia, fever, and/or CRP elevation improved sensitivity without loss of specificity in a population at high risk of TB-IRIS. We recommend that in future studies on TB-IRIS a version of the INSHI case definition with objective measure be used, next to the traditional case definition. CRP appears to be promising as a test for ruling out TB-IRIS.

Acknowledgements

We like to thank Bart K. Jacobs for constructing figure 2.1.

Soluble inflammatory markers and LTA4H genotype in tuberculosis-associated IRIS and the effect of prophylactic prednisone

Introduction

Paradoxical tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) complicates treatment in 18% (95%CI 16-21%) of patients receiving treatment for HIV-associated tuberculosis (TB). This immunopathological reaction results in new, recurrent, or worsening signs or symptoms of TB and usually occurs within the first few weeks after starting antiretroviral therapy (ART) [18]. TB-IRIS can be treated with prednisone, which leads to less hospitalizations and therapeutic interventions and a more rapid improvement of symptoms and chest radiograph abnormalities, and a more rapid decrease of C-reactive protein (CRP) [37]. We have recently completed a randomized, double-blind, placebo-controlled trial, the PredART trial [43, 137]. The primary objective was to determine whether prednisone is effective in preventing the development of TB-IRIS. Participants identified as being at high risk for paradoxical TB-IRIS received either a 28-day course of prophylactic prednisone or identical placebo within 48 hours after starting antiretroviral therapy. TB-IRIS diagnosis was adjudicated by an independent committee using the International Network for the Study of HIV-associated IRIS (INSHI) criteria [27]. We found prednisone reduces the risk of TB-IRIS by 30%. How prednisone reduces the risk of TB-IRIS is not clear. It is unlikely that prednisone only suppresses TB-IRIS symptoms: the median time to TB-IRIS (in participants developing TB-IRIS) was similar in both the prednisone and the placebo arm; moreover, prednisone also reduced the incidence of more severe TB-IRIS, judged from the number of patients with TB-IRIS fulfilling at least one INSHI major criterion or receiving prednisone treatment for TB-IRIS. Also, there did not appear to be "breakthrough cases" when the 28-day course of prednisone was stopped suggesting that the course of prednisone had prevented the onset of the syndrome, rather than merely suppressing or delaying symptoms.

TB-IRIS associates with hypercytokinemia [59]. The immunopathological mechanisms underlying it are not completely understood. Patients with TB-IRIS show expansion of interferon-γ (IFN-γ) producing Th1 cells, but as this expansion is not present in all patients with TB-IRIS, and also occurs in some patients without it, it seems unlikely that this association is causal. The same holds true for other T cell subsets [138]. Moreover, neutrophils, natural killer (NK)-cells, and monocytes/macrophages have all been associated with TB-IRIS [138]. Transcriptomic profiling of whole blood of patients with TB-IRIS shows

involvement of the innate immune system: inflammation in TB-IRIS occurs early and innate immune signaling and activation of the inflammasome appear to be early events, resulting in upregulation of cytokines and chemokines [67]. Prednisone affects several cytokines and chemokines in patients with HIV-associated TB: treatment of TB-IRIS with prednisone was shown to reduce the serum concentrations of interleukin (IL)-6, IL-10, IL-12p40, IFN-γ, C-X-C motif chemokine ligand (CXCL)-10 (IFN-γ induced protein 10 (IP-10)), and tumor-necrosis factor-α (TNF-α) [139]; adjunctive corticosteroid treatment for TB before starting ART was associated with lower pre-ART concentrations of these same cytokines as well as CXCL-8 (IL-8) and IL-18 [63]. In the latter study, however, patients also differed from their non-corticosteroid controls in the severity and type of TB, which may have confounded the observed association.

A recent study suggests that the efficacy of corticosteroids as adjunctive treatment in TB might depend on *lta4h* genotype [96]. LTA4 hydrolase (LTA4H) is an enzyme that hydrolyses leukotriene (LT) A4 into the pro-inflammatory LTB4 [97]. It is regulated by a single nucleotide polymorphism (SNP) close to the promoter region of the *lta4h* gene [98]. The wildtype genotype (CC) is associated with lower concentrations of LTA4H, whereas the double mutant genotype (TT) is associated with increased LTA4H activity. Several studies have evaluated the effect of *lta4h* polymorphism on outcomes in TB. The initial study, including patients from Vietnam, showed that heterozygosity (intermediate LTA4H expression) is associated with protection from both pulmonary and meningeal TB and results in fewer deaths from TB meningitis (TBM). Moreover, only those with the hyperinflammatory TT genotype benefitted from treatment with corticosteroids for TBM [96]. Other studies assessing *lta4h* genotype and TB found different results: the association with pulmonary TB could not be shown in cohorts from Russia [140], China [141], and Mozambique [142]. Results for TBM are also equivocal: heterozygosity - in the same cohort from China - was associated with less extrapulmonary TB, including TBM [141], but no association with TBM or its outcome was found in an Indonesian cohort [143]. A more recent Vietnamese cohort study showed survival benefit in TBM for those with the TT genotype, but only in HIV uninfected patients. HIV-infection was a risk factor for death, independent from *lta4h* genotype [144]. None of the other studies included HIV infected patients in their analysis. In all studies, except the initial study from Vietnam, all patients were treated with dexamethasone.

The increased production of pro-inflammatory cytokines in mutant genotypes as a result of increased LTB4 production, especially the hyperinflammatory TT genotype, could potentially play a role in the development of TB-IRIS. One study evaluated *lta4h* genotype in TB-IRIS: they found no effect of genotype on the incidence of TB-IRIS in an Indian cohort of 142 patients with newly diagnosed TB and low CD4 cell counts. However, severe IRIS (defined as a Karnofsky score of 50 or less or a clinical condition mandating hospitalization or prolonging of hospital admission) was more common in those with mutant genotypes (CT

and TT) compared to those with wildtype (CC) [99]. In contrast to the findings from the TBM study in Vietnam [96], steroids were effective in treating TB-IRIS in all genotypes [99].

In this study, we aimed to further investigate the immunological mechanisms underlying TB-IRIS and the mechanisms by which prednisone is effective in preventing it. Moreover, we assessed the role of *lta4h* genotype in relation to the risk of TB-IRIS and the efficacy of prophylactic prednisone. Specifically, we wanted to assess the following:

1. The association between (concentrations of) cytokines/chemokines and (the development of) TB-IRIS in patients with HIV-associated TB, at baseline and two weeks after starting ART.
2. The effect of prednisone use on concentrations of cytokines/chemokines in patients with HIV-associated TB two weeks after starting ART.
3. The effect of prednisone use on the change in concentrations of cytokines/chemokines in patients predicted to develop TB-IRIS (using a baseline predictive model for TB-IRIS including cytokines/chemokines and other patient characteristics, restricted to participants in the placebo arm). We compared concentrations of cytokines/chemokines at week 2 (two weeks after starting ART) in the prednisone vs the placebo arm in those predicted to develop TB-IRIS, regardless of whether they developed TB-IRIS or not; and also compared cytokines/chemokines at week 2 in those predicted to develop TB-IRIS in the prednisone arm with those not predicted to develop TB-IRIS in the placebo arm.
4. The association between LTA4H genotype and the development of TB-IRIS overall and in the placebo arm.
5. The association between LTA4H genotype and cytokine/chemokine profiles in patients with HIV-associated TB, at baseline and two weeks after starting ART.
6. The interaction between LTA4H genotype and the effect of prednisone in preventing TB-IRIS in patients with HIV-associated TB.

Methods

Study design, participants and setting

This study was a substudy of the PredART trial [43]. Participants were recruited between August 2013 and February 2016 from four different clinics in Khayelitsha, Cape Town, South Africa. Eligible patients were adults, HIV positive with a CD4 count < 100 cells/µl and ART naïve; they were recently diagnosed with TB, either microbiologically confirmed or clinically diagnosed with a good response to treatment and had been receiving antituberculosis treatment for 30 days or less. Only participants who took at least one dose of ART were included in our substudy.

The substudy was approved by the same ethical committees that approved the main trial. By enrolling in the Pred-ART trial participants agreed to continued storage of their samples for future independent research with ethical permission. Separate informed consent for genetic testing was obtained from all but one participant who was excluded from genotyping in this substudy.

Procedures

Plasma was isolated and stored at -80 degrees for every participant enrolled in the trial at week 0 (start of ART), 2, 4, and 12; additional samples were taken and stored at the time of suspected TB-IRIS. For this study, we used samples taken at week 0 and week 2.
We measured plasma basic fibroblast growth factor (FGF), eotaxin, granulocyte colony-stimulating factor (G-CSF), granulocyte- macrophage colony-stimulating factor (GM-CSF), IFN-γ, IL-1β, IL-1 receptor antagonist (ra), IL-2, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12p70, IL-13, IL-15, Il-17, C-C motif chemokine ligand (CCL)-2 (monocyte chemoattractant protein (MCP)-1), CCL-3 (macrophage inflammatory protein (MIP)-1α), CCL-4 (MIP-1β), CCL-5 (regulated on activation, normal T cell expressed and secreted (RANTES)), CXCL-8, CXCL-10, platelet-derived growth factor (PDGF), TNF-α, and vascular endothelial growth factor (VEGF) using Bio-Plex Pro™ Human Cytokine 27-plex Assay (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions . IL-18, soluble (s) tissue factor (TF), sCD14, and sCD163 were measured using standardized enzyme-linked immunosorbent assays (ELISAs) (R&D systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Where cytokines measured by Luminex had a value out of range below (or above) the detection limit, the value was replaced with the mean of the lowest (or highest) measurable values across all plates. For cytokines measured by ELISA, the lowest (or highest) standard of that plate was taken, divided by (or multiplied by) 2. Cytokines with more than 70% of the measurements below the detection limit were excluded from analysis.

DNA was extracted from whole blood using QIAamp® DNA Blood Midi Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions and stored at -20 degrees. *Lta4h* gene promoter region SNP (rs17525495) was analyzed from extracted DNA using singleplex snapshot PCR (Thermo Fisher, Waltham, MA, USA). Fragments were resolved on an ABI 3130xl Genetic Analyzer and the data analysed on GeneMapper, Bioedit Sequence Alignment Editor v7.2.5 23, and Finch TV v1.4.0 [145].

Statistical analysis

Cytokines and chemokines concentrations are presented as medians with interquartile ranges. Correction for batch effect was done for all cytokines/chemokines using limma and removeBatchEffect in R. In short, this package adjusts for confounding by batch effect by fitting a linear model to the data including the batches and then removing the component due to batch effect. LTA4H genotype is presented as wildtype (CC) and mutant (CT and TT). Consistency of the observed genotypes with the Hardy-Weinberg equilibrium was tested

using the Pearson Chi square test. Cross-sectional comparisons of cytokines/chemokines comparing participants with and without TB-IRIS or participants in prednisone and placebo arms was done using the Wilcoxon rank sum test. Comparisons between different time points were done by Wilcoxon signed rank test. Analyses were adjusted for multiple comparisons using the Bonferroni correction (n = 26), a p-value of < 0.002 was considered significant. For the comparisons between the prednisone and the placebo arms, those in the placebo arm who started open label prednisone treatment for TB-IRIS before the week 2 sample was taken (n = 5) were excluded from the analysis. Association between LTA4H genotype and development of TB-IRIS was assessed using the Pearson Chi square test. The effect of LTA4H genotype on the efficacy of prophylactic prednisone was assessed using Cox proportional hazard models, and represented in Kaplan-Meier plots, with time to TB-IRIS as outcome and treatment arm, genotype and their interaction as variables. A predictive model for TB-IRIS was developed using only the participants from the placebo arm. The following baseline parameters were included in the model: 26 cytokines/chemokine concentrations, CD4 count, HIV viral load, hemoglobin, neutrophils, urine LAM, and CRP. A series of simple logistic regression models with each of the candidate independent variables was fitted. Next, a multiple logistic regression model was fitted including all individual variables with a p-value < 0.20. One at a time the variables whose p-value in the multiple regression model was > 0.10 were removed, starting from the one with the highest p-value.

Results

Study participants

Between August 2013 and February 2016, 240 participants were enrolled in the PredART trial. Two participants never started ART and were excluded from the analyses. Of the remaining 238, 181 had plasma available for analysis; plasma for 57 participants could not be used due to an error when processing the samples: samples were randomly diluted when extracting peripheral blood mononuclear cells from the same tubes, without recording the dilution factor. LTA4H genotyping was done in 213 participants; six participants had no blood stored for DNA extraction for logistic reasons, genotyping was unsuccessful in 18 participants. Baseline characteristics of the 181 participants with plasma available are summarized in Table 3.1A; those for the 213 participants with genotyping done in Table 3.1B. These are comparable to the baseline characteristics of the entire PredART cohort. Seventy-two participants (40%) developed TB-IRIS at a median of 9.5 days (IQR 6-13 days) after starting ART.

G-CSF, GM-CSF, IL-5, IL-15 and VEGF all had lower than detectable values in more than 70% of the participants and were excluded from the analysis.

TABLE 3.1A Baseline characteristics for participants for whom plasma was available for analysis (n = 181).

	Prednisone arm (n = 93)		Placebo arm (n = 88)		TB-IRIS (n = 72)		No TB-IRIS (n = 109)	
Age	37	(31 – 42)	37	(29-43)	35	(29-41)	38	(31-44)
Male sex	57	(61%)	56	(64%)	48	(67%)	65	(60%)
CD4 count (cells/µl)	51	(24-84)	55	(25-90)	39	(23-60)	67	(28-102)
HIV viral load (log_{10} cp/ml)	5.5	(5.2-5.9)	5.6	(5.2-5.9)	5.5	(5.3-5.9)	5.5	(5.2-5.9)
Time between start TB-Rx and start ART (d)	16	(14-21)	17	(15-21)	16	(14-21)	17	(15-21)
Prednisone arm	93	(100%)	0	(0%)	30	(42%)	63	(58%)
TB-IRIS	30	(32%)	42	(48%)	72	(100%)	0	(0%)
Time to TB-IRIS (d)	10	(7-13)	8	(6-13)	9.5	(6-13)		

d = days; cp = copies. Data are shown as number (percentage) or median (interquartile range).

TABLE 3.1B Baseline characteristics for participants for whom LTA4H genotyping was done (n = 213).

	CC (n = 173)		CT/TT (n = 40)	
Age	36	(29-43)	38	(33-43)
Male sex	105	(61%)	22	(55%)
CD4 count (cells/µl)	49	(26-84)	47	(18-99)
HIV viral load (log_{10} cp/ml)	5.5	(5.2-5.9)	5.7	(5.1-5.9)
Time between start TB-Rx and start ART (d)	17	(15-22)	16	(14-19)
Prednisone arm	85	(49%)	19	(48%)
TB-IRIS	67	(39%)	16	(40%)
Time to TB-IRIS (d)	8	(5-13)	11	(7-13)

d = days; cp = copies. Data are shown as number (percentage) or median (interquartile range).

There was no difference in cytokine profile at baseline between participants who developed TB-IRIS and those who did not.

When comparing individual cytokines at baseline between participants who developed TB-IRIS and those who did not (n = 172, participants in both prednisone and placebo arm included), we did not find an association between plasma concentrations of any of the cytokines/chemokines and the development of TB-IRIS (Table 3.2).

TABLE 3.2 Concentrations of cytokines and chemokines at week 0 and week 2 in all participants with and without TB-IRIS

	TB-IRIS					No TB-IRIS					p (IRIS vs no IRIS)	
	Week 0 (n = 70)		Week 2 (n = 58)		p	Week 0 (n = 102)		Week 2 (n = 98)		p	Week 0	Week 2
IL-1b	0.13	(0.11 - 0.14)	0.14	(0.12-0.18)	***0.0003***	0.12	(0.11-0.16)	0.12	(0.11-0.14)	0.68	0.74	0.009
IL-18	1139.1	(841.5 - 1792.5)	1004.6	(609.6-1626.8)	0.02	1057.9	(723.6-1815.6)	786.5	(546.5-1333.5)	***<0.0001***	0.44	0.12
IL-1ra	110.3	(67.45 - 175.1)	226.6	(102.4-530.1)	***0.0001***	121.3	(79.6-267.8)	112.8	(63.0-233.3)	0.07	0.34	***0.0002***
IL-6	0.10	(0.09 - 0.21)	0.18	(0.10-0.52)	0.004	0.10	(0.07-0.79)	0.10	(0.07-0.38)	0.55	0.99	0.06
TNF	15.0	(10.1 - 22.1)	22.6	(16.4-34.0)	***<0.0001***	15.1	(10.5-25.2)	13.6	(8.6-29.6)	0.66	0.96	***0.0007***
IFN-y	0.41	(0.32 - 0.77)	0.36	(0.20-0.64)	0.01	0.36	(0.36-0.98)	0.36	(0.11-0.56)	***0.0001***	0.88	0.19
IL-12p70	0.34	(0.25 - 0.37)	0.35	(0.25-0.4)	0.20	0.33	(0.25-0.50)	0.34	(0.25-0.45)	0.16	0.91	0.97
IL-17	0.46	(0.25 - 0.61)	0.46	(0.34-0.65)	0.53	0.46	(0.34-0.52)	0.46	(0.34-0.56)	0.60	0.93	0.47
IL-2	1.21	(0.93 - 1.74)	1.41	(1.14-2.25)	***0.0013***	1.03	(0.62-1.74)	1.00	(0.62-1.99)	0.61	0.14	***0.0003***
IL-7	1.47	(1.37 - 2.20)	1.47	(1.40-2.01)	0.83	1.40	(1.03-1.59)	1.40	(1.37-2.01)	0.02	0.03	0.43
IL-4	0.15	(0.08 - 0.37)	0.14	(0.10-0.33)	0.07	0.17	(0.08-0.69)	0.14	(0.08-0.66)	0.03	0.34	0.23
IL-9	24.0	(20.3 - 30.1)	23.3	(19.1-28.3)	0.11	25.8	(21.3-32.7)	24.7	(20.6-32.3)	0.07	0.19	0.23
IL-13	0.05	(0.05 - 0.07)	0.05	(0.05-0.07)	0.22	0.05	(0.05-0.08)	0.05	(0.05-0.07)	0.54	0.79	0.93
IL-10	0.41	(0.31 - 0.61)	0.41	(0.31-0.81)	0.32	0.41	(0.24-0.55)	0.41	(0.31-0.58)	0.43	0.23	0.13
CCL2	16.5	(6.3 - 26.1)	8.19	(2.70-18.53)	***<0.0001***	13.2	(6.5-27.0)	8.97	(3.61-19.80)	***<0.0001***	0.72	0.52
CCL3	2.05	(1.21 - 4.03)	3.40	(1.77-7.56)	***0.001***	1.92	(1.22-3.21)	1.99	(1.15-4.50)	0.12	0.50	***0.0011***
CCL4	24.5	(19.5 - 29.9)	28.6	(23.2-43.5)	***0.001***	25.2	(21.3-31.7)	25.3	(20.5-33.4)	0.80	0.42	0.01
CCL5	1486.5	(385.4 - 3287.8)	1208.1	(510.4-2833.9)	0.02	1331.2	(719.7-2334.3)	1025.8	(474.2-1587.4)	0.003	0.92	0.14
CCL11	7.01	(4.34 - 15.2)	6.47	(3.81-11.91)	***0.0016***	9.89	(4.95-20.68)	9.49	(5.07-22.61)	0.28	0.17	0.01
CXCL8	1.26	(1.06 - 3.75)	1.75	(1.06-5.70)	0.005	1.40	(0.95-3.73)	1.12	(0.64-3.73)	0.56	0.72	0.07
CXCL10	997.2	(424.8 - 1594.3)	1083.0	(412.1-1624.4)	0.31	811.6	(430.5-1561.0)	488.7	(258.9-983.1)	***<0.0001***	0.72	***0.0017***
PDGF	22.5	(14.2 - 47.1)	24.6	(16.2-55.3)	0.83	19.9	(11.6-50.3)	20.1	(12.3-49.15)	0.50	0.60	0.48
basic FGF	2.04	(1.29 - 2.77)	2.04	(1.42-2.93)	0.31	1.99	(1.29-3.40)	1.77	(1.29-2.77)	0.33	0.78	0.12
CD14	4578.3	(3413.9 - 5401.8)	4485.0	(3539.8-5635.6)	0.12	4319.4	(3379.9-5658.1)	4168.0	(2932.9-5409.2)	0.08	0.71	0.13
CD163	1043.5	(788.9 - 1501.0)	1170.5	(744.8-1655.6)	0.02	1064.4	(813.6-1462.9)	1057.3	(739.3-1479.0)	0.50	0.79	0.20
TF	38.2	(30.0 - 46.6)	38.7	(30.6-51.6)	0.28	36.7	(30.7-43.6)	38.8	(30.9-46.5)	0.10	0.97	0.82

Data are shown as median (interquartile range). Concentrations (pg/ml) between participants with and without TB-IRIS were compared using the Wilcoxon rank sum test; concentrations between week 0 and week 2 were compared using the Wilcoxon signed rank test (56 pts with TB-IRIS vs 91 pts without TB-IRIS). After adjusting for multiple comparisons using the Bonferroni correction, a p-value of < 0.002 was considered significant.

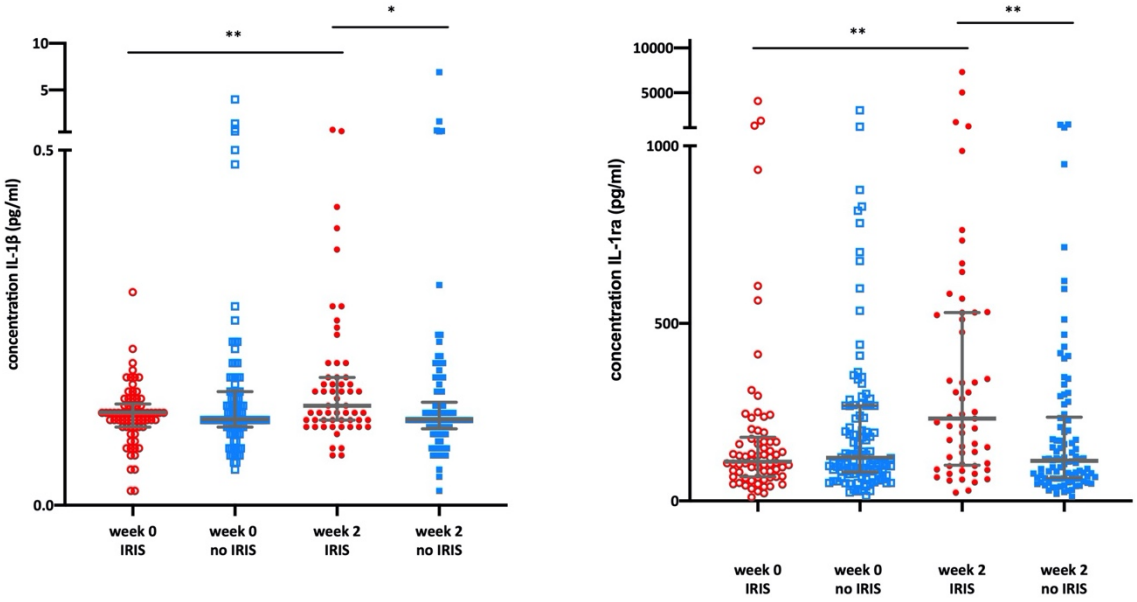
TABLE 3.3 Concentrations of cytokines and chemokines at week 0 and week 2 in participants with and without TB-IRIS – Placebo arm only

	TB-IRIS					No TB-IRIS					p (IRIS vs no IRIS)	
	Week 0 (n = 37)		Week 2 (n = 32)		p	Week 0 (n = 45)		Week 2 (n =40)		p	Week 0	Week 2
IL-1b	0.13	(0.11-0.15)	0.16	(0.12-0.25)	0.005	0.12	(0.11-0.14)	0.12	(0.11-0.18)	0.60	0.70	0.07
IL-18	1291.1	(886.5-2577.5)	1045.8	(758.1-2414.6)	0.06	1036.3	(793.2-1815.6)	871.4	(566.3-1583.7)	0.03	0.18	0.23
IL-1ra	140.4	(68.9-233.7)	335.8	(197.4-613.9)	***0.0016***	127.7	(79.6-234.1)	122.8	(70.7-336.0)	0.70	0.95	0.0022
IL-6	0.10	(0.09-0.18)	0.31	(0.10-0.62)	***0.0002***	0.10	(0.09-1.40)	0.21	(0.09-1.89)	0.95	0.21	0.47
TNF	18.2	(12.4-24.4)	23.9	(19.5-35.8)	***< 0.0001***	14.7	(10.5-25.2)	17.7	(9.4-36.0)	0.15	0.46	0.02
IFN-y	0.50	(0.33-0.93)	0.36	(0.22-0.71)	0.03	0.36	(0.36-0.85)	0.36	(0.20-0.56)	0.24	0.33	0.55
IL-12p70	0.34	(0.25-0.36)	0.36	(0.31-0.40)	0.05	0.33	(0.25-0.50)	0.34	(0.25-0.42)	0.54	0.92	0.45
IL-17	0.46	(0.34-0.65)	0.54	(0.34-0.83)	0.20	0.46	(0.34-0.50)	0.46	(0.34-0.74)	0.90	0.84	0.29
IL-2	1.33	(0.94-1.84)	1.70	(1.22-2.27)	0.004	1.00	(0.62-1.57)	0.96	(0.62-1.65)	0.60	0.09	0.0024
IL-7	1.47	(1.40-2.57)	1.47	(1.40-2.15)	0.56	1.40	(1.03-1.47)	1.40	(1.03-1.47)	0.27	0.0024	0.02
IL-4	0.21	(0.13-0.54)	0.19	(0.13-0.48)	0.09	0.14	(0.08-0.72)	0.14	(0.08-0.60)	0.01	0.79	0.67
IL-9	25.6	(21.6-32.0)	25.7	(22.7-29.7)	0.16	25.2	(19.4-32.1)	25.5	(20.8-32.8)	0.59	0.95	0.96
IL-13	0.05	(0.05-0.07)	0.05	(0.05-0.06)	0.20	0.05	(0.05-0.06)	0.05	(0.05-0.07)	0.18	0.60	0.76
IL-10	0.41	(0.31-0.62)	0.41	(0.32-1.09)	0.16	0.31	(0.24-0.41)	0.41	(0.24-0.42)	0.32	0.02	0.02
CCL2	16.7	(7.2-38.7)	8.54	(2.83-19.04)	***0.0005***	13.2	(8.5-27.0)	9.31	(4.84-18.81)	0.03	0.35	0.43
CCL3	2.08	(1.21-3.85)	3.56	(1.87-7.90)	0.02	1.73	(1.22-2.98)	2.07	(1.50-4.72)	***0.0005***	0.17	0.04
CCL4	24.7	(21.5-30.0)	29.8	(26.2-46.6)	0.005	24.4	(20.4-28.6)	26.9	(21.6-33.8)	0.007	0.31	0.03
CCL5	1474.6	(476.2-3106.9)	1307.2	(608.6-2362.1)	0.49	1089.5	(608.8-2241.0)	1006.1	(464.0-1746.2)	0.69	0.89	0.25
CCL11	9.51	(4.34-16.63)	6.67	(3.98-15.53)	0.03	8.05	(4.53-19.43)	9.53	(4.82-18.63)	0.07	0.77	0.18
CXCL8	1.50	(1.06-4.33)	2.02	(1.06-11.52)	0.02	1.06	(0.21-3.73)	1.32	(0.55-5.06)	0.52	0.17	0.09
CXCL10	1009.7	(572.7-1690.6)	1208.9	(707.1-1904.6)	0.22	845.4	(298.7-1662.1)	643.8	(278.5-1348.4)	0.13	0.48	0.015
PDGF	20.6	(14.2-48.8)	25.6	(16.2-53.3)	0.50	17.6	(11.6-36.9)	28.6	(15.1-74.4)	0.02	0.64	0.39
basic FGF	2.07	(1.29-2.77)	2.04	(1.37-3.01)	0.52	1.94	(1.29-2.61)	1.51	(1.29-2.69)	0.75	0.33	0.18
CD14	4848.1	(3441.0-5605.9)	5020.6	(4034.5-6305.2)	0.10	4521.7	(3640.9-5658.1)	4693.6	(3417.2-5491.9)	0.56	0.79	0.18
CD163	1040.2	(820.1-1526.1)	1260.9	(719.5-1889.8)	0.08	1101.6	(881.4-1382.1)	1085.7	(840.2-1509.8)	0.33	0.86	0.32
TF	39.3	(31.8-45.6)	39.6	(30.8-48.3)	0.94	36.9	(30.7-43.6)	37.2	(30.6-44.4)	0.34	0.51	0.63

Data are shown as median (interquartile range). Concentrations (pg/ml) between participants in the placebo arm with and without TB-IRIS were compared using the Wilcoxon rank sum test; concentrations between week 0 and week 2 were compared using the Wilcoxon signed rank test (31 pts with TB-IRIS vs 39 pts without TB-IRIS). After adjusting for multiple comparisons using the Bonferroni correction, a p-value of < 0.002 was considered significant.

TB-IRIS is associated with higher concentrations of several chemo- and cytokines at week 2

Plasma concentrations of IL-1b, IL-1ra, TNF-α, IL-2, CCL-3, CCL-4, CCL-11 and CXCL-10 were higher 2 weeks after starting ART in participants who developed TB-IRIS (n = 156, prednisone and placebo arms combined) compared to those who did not. After correction for multiple comparisons, IL-1ra, IL-2, TNF, CCL-3 and CXCL-10 remained significant (Table 3.2 and Figure 3.1). When evaluating the change over time of these cytokines, we found that plasma concentrations of IL-1ra, IL-2, TNF-α and CCL-3 increase from week 0 to week 2 in participants with TB-IRIS, while no increase is seen in participants without TB-IRIS. Concentrations of IFN-γ and CXCL-10 decrease between week 0 and week 2 in participants without TB-IRIS, while remaining unchanged in participants with TB-IRIS.
Repeating the analyses only including participants in the placebo arm showed similar results, although due to smaller numbers most cytokines lost statistical significance (Table 3.3).



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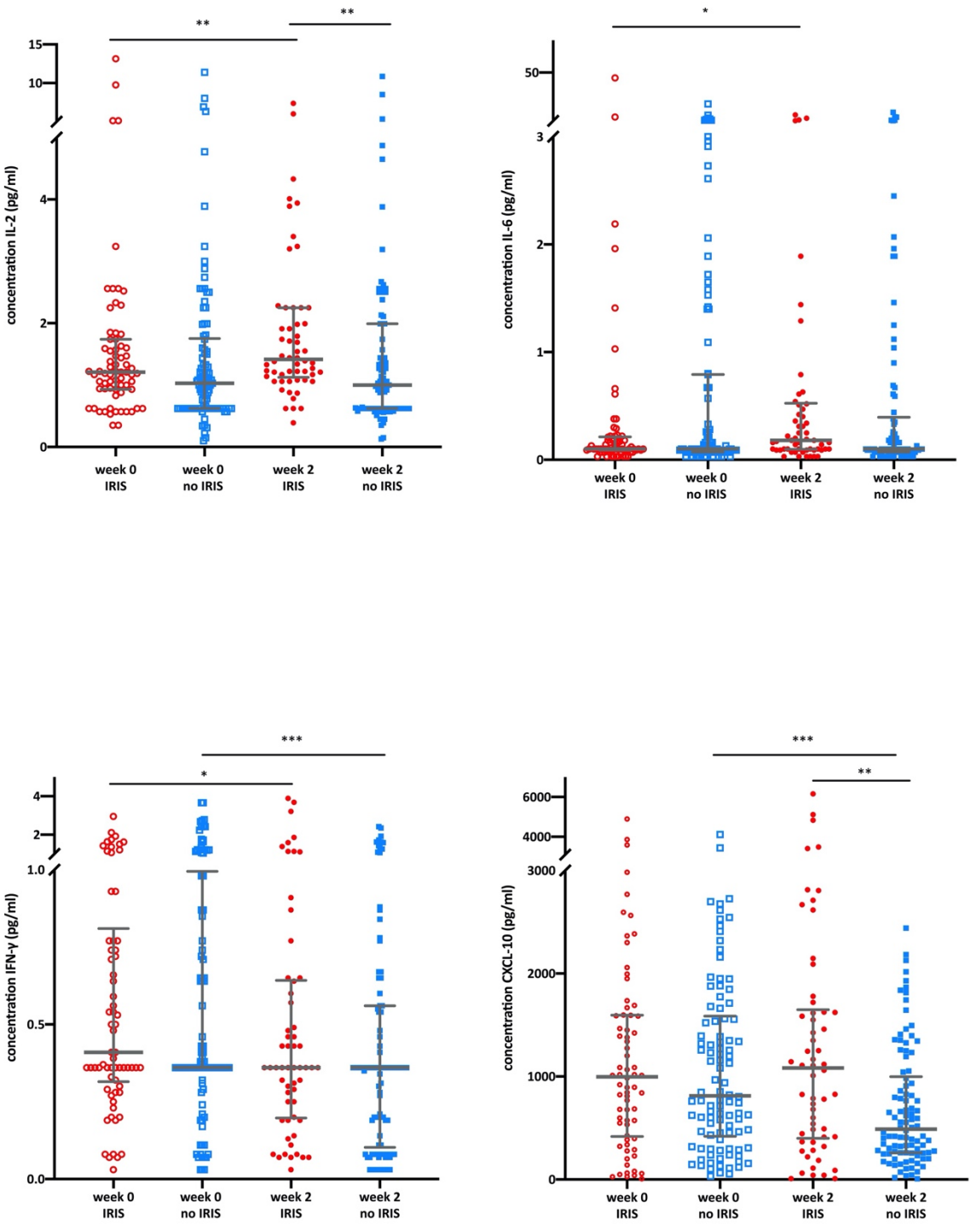

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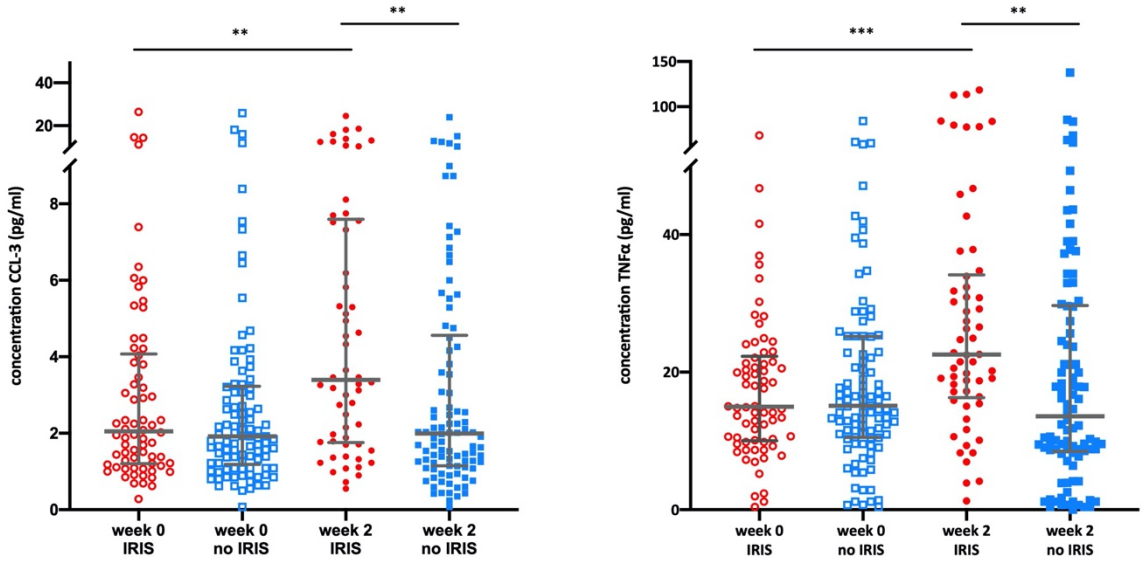
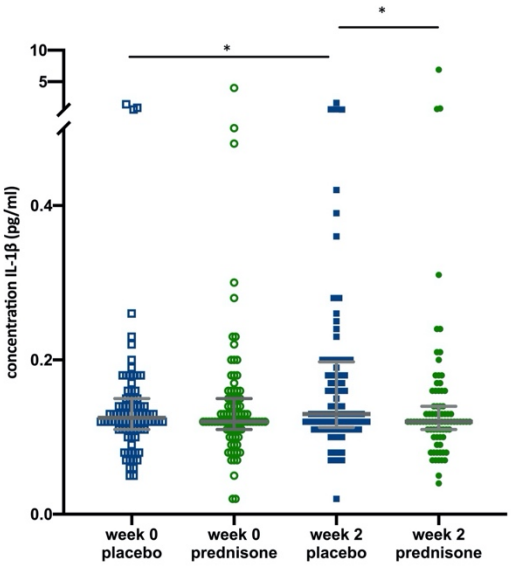


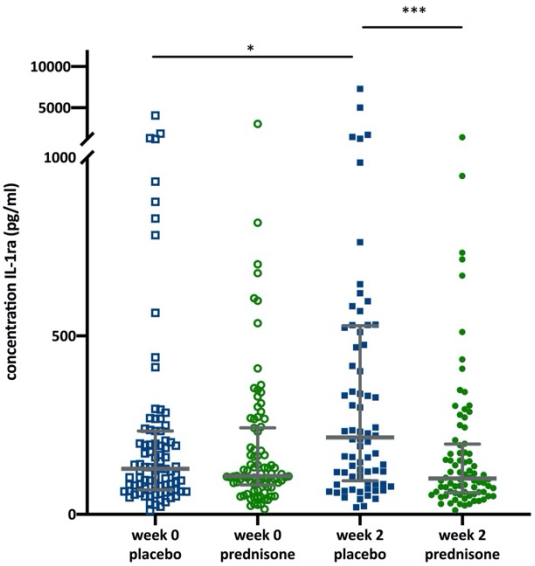
FIGURE 3.1 Absolute concentrations of selected cytokines and chemokines at week 0 and week 2 in participants who did and did not develop TB-IRIS.

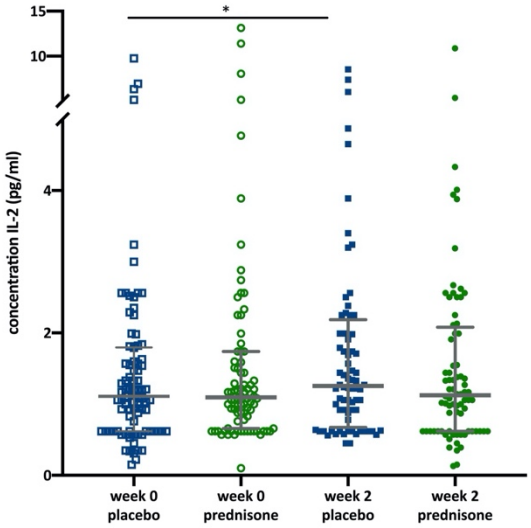
Concentrations (pg/ml) were compared between participants with and without TB-IRIS using the Wilcoxon rank sum test, and between week 0 and week 2 using the Wilcoxon signed rank test. After adjusting for multiple comparisons using the Bonferroni correction, p-value of < 0.002 was considered significant. * < 0.05 ** < 0.002 *** < 0.0002

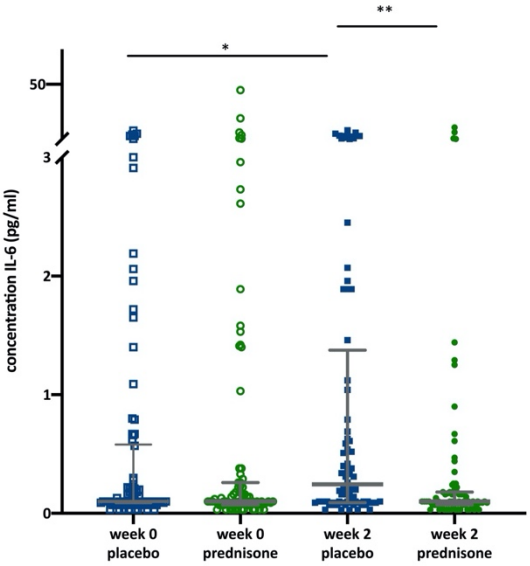
Prednisone reduces plasma concentrations of several chemo- and cytokines

We compared plasma concentrations of chemo- and cytokines between participants randomized in the prednisone arm and those in the placebo arm, regardless whether they developed TB-IRIS. We found prednisone was associated with lower concentrations of IL-1β, IL-18, IL-1ra, IL-6, TNF-α, IL-17, CCL-3, CCL-4, CXCL-10 and sCD14, and at week 2; IL-1ra, IL-6 and CXCL-10 remained significant after correction for multiple comparisons (Table 3.4). Prednisone was associated with a decrease in concentration of CXCL-10, (as well as IFN-γ , IL-18 and CCL-5) between week 0 and week 2, whereas concentrations of those cytokines remained unchanged over time in the placebo arm. In the placebo arm, IL-1ra, IL-6 (although no longer significant after correction for multiple comparisons), TNF-α and CCL-3 (as well as CCL-4) increased over time, while no increase in these analytes was seen in the prednisone arm. IL-18 and CCL-2 decreased over time in both arms (Table 3.4 and Figure 3.2).









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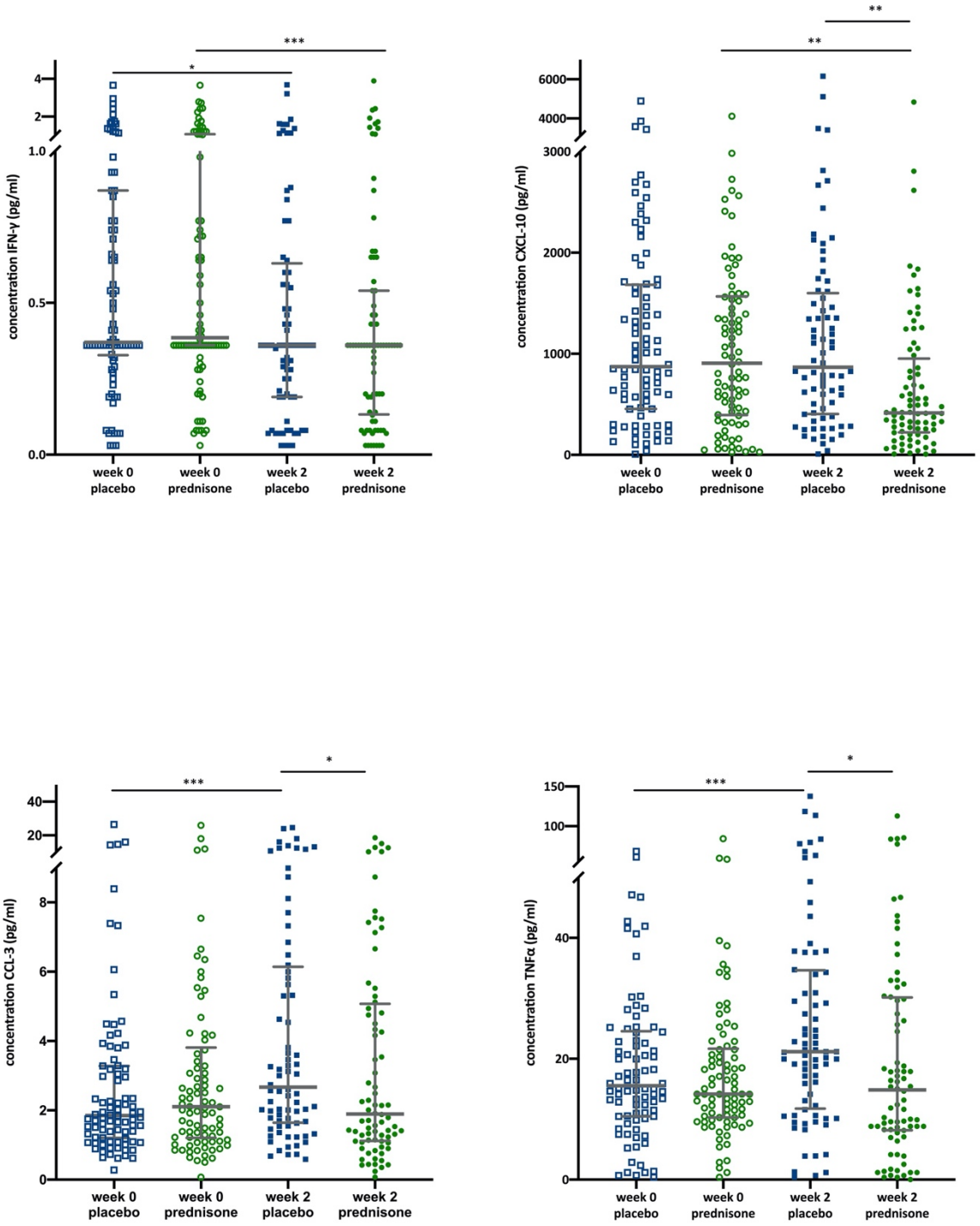


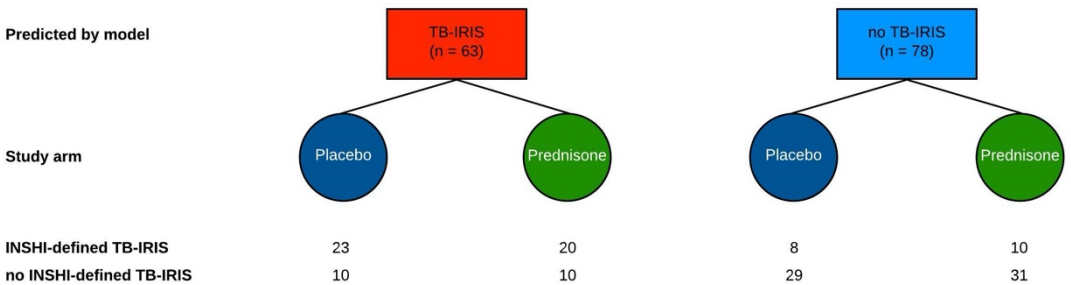
FIGURE 3.2 Concentrations of selected cytokines and chemokines at week 0 and week 2 in participants in prednisone and placebo arms.

Concentrations were compared between participants in the prednisone and the placebo arm using the Wilcoxon rank sum test, and between week 0 and week 2 using the Wilcoxon signed rank test. After adjusting for multiple comparisons using the Bonferroni correction, p-value of < 0.002 was considered significant. * < 0.05 ** < 0.002 *** < 0.0002

We developed a prediction model in participants randomized into the placebo arm to identify participants at baseline who would develop TB-IRIS in our cohort. We used the model to identify participants likely to develop TB-IRIS in both the prednisone and the placebo arm and reasoned that in those participants identified at risk in the prednisone arm, subsequently not developing TB-IRIS could be attributed to prednisone. The final model included baseline CD4 count, Hb, IL-6, IL-12p70, and CCL3. We defined a participant to be likely to develop TB-IRIS if the model-predicted probability of TB-IRIS was > 0.5. The model predicted TB-IRIS in the placebo group (n = 85) with a receiver operator curve area under the curve of 0.84 (Figure 3.3). Because these analyses are more exploratory in nature, we did not correct for multiple comparisons and considered a p-value of < 0.05 to be significant.

Using the model, we identified 63/141 participants (with plasma available for analysis at both week 0 and week 2) likely to develop TB-IRIS, 30 in the prednisone and 33 in the placebo arm (excluding participants who used open label prednisone before the time of taking week 2 blood (n = 5). To validate the model, we first compared participants predicted to develop TB-IRIS with those not predicted to develop TB-IRIS in the placebo arm: concentrations of IL-1ra, TNF-α and CCL-3 were significantly higher at week 2 in participants predicted to develop TB-IRIS, in line with our findings from the analysis of those who did develop INSHI-defined TB-IRIS; we also found higher concentrations of IL-18 and sCD14 at week 2 in participants predicted to develop TB-IRIS, whereas concentrations of IL-7 were lower in these participants (Table 3.5 and Figure 3.4A). Assessing the effect of prednisone vs placebo in participants predicted to not develop TB-IRIS showed significantly lower concentrations of IL-1ra and IL-6 in the prednisone arm at week 2 (Table 3.5 and Figure 3.4B), also in line with our analyses of prednisone vs placebo in the entire study cohort. When assessing the effect of prednisone on concentrations of chemokines and cytokines in the entire study cohort, however, its effect on modulating the mediators of TB-IRIS might be

A



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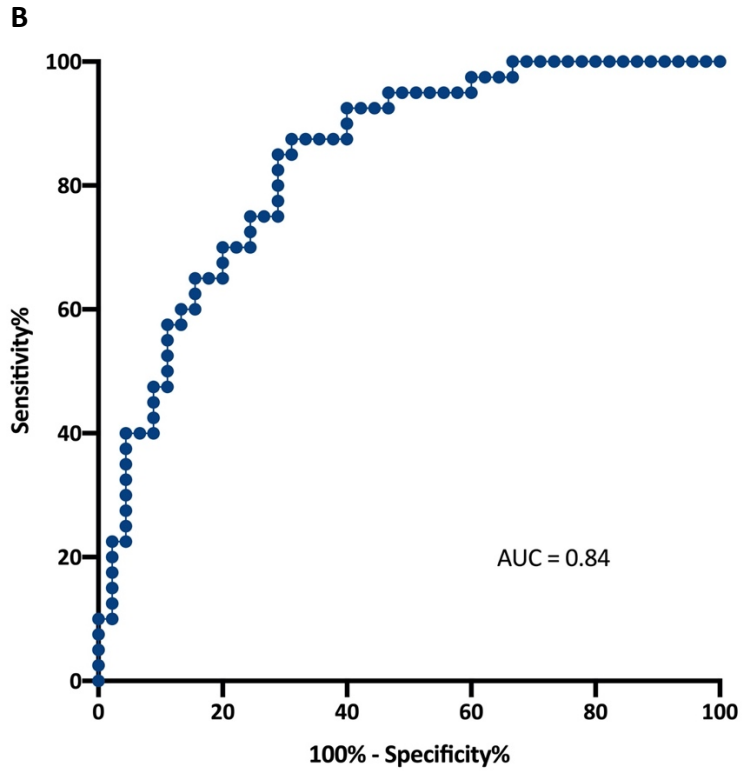

FIGURE 3.3 Performance of the model predicting the development of TB-IRIS in trial participants.

A. Number of participants predicted to develop TB-IRIS and not predicted to develop TB-IRIS by the model and their outcome defined by the INSHI criteria by study arm.
B. Receiver operator curve area under the curve of the model predicting the development of TB-IRIS in participants in the placebo arm at baseline.
To construct the model, first a series of simple logistic regression models with baseline values of each of the following independent variables was fitted: the 26 chemokines/cytokines listed in tables 3.2-6, CD4 count, HIV viral load, hemoglobin, neutrophils, urine LAM, and CRP. Next, a multiple logistic regression model was fitted including all individual variables with a p-value < 0.20. One at a time the variables whose p-value in the multiple regression model was > 0.10 were removed, starting from the one with the highest p-value. The final model included baseline CD4 count, Hb, IL-6, IL-12p70, and CCL-3. We defined a participant to be likely to develop TB-IRIS if the model-predicted probability of TB-IRIS was > 0.5.

diluted because prednisone was also given to participants who would never develop TB-IRIS. Therefore, we next compared concentrations of chemo- and cytokines in participants predicted to develop TB-IRIS between prednisone and placebo arms. We found lower concentrations at week 2 of IL-1β, IL-1ra, IL-6, TNF and CXCL-10 in participants in the prednisone arm (Table 3.5 and Figure 3.4C). Lastly, we tested our hypothesis that prednisone treatment modifies the cytokine profile of participants at risk of developing TB-IRIS to resemble the cytokine profile of participants who did not develop TB-IRIS at week 2. We compared chemo- and cytokine concentrations of participants predicted to develop TB-IRIS receiving prednisone with those of participants predicted to not develop TB-IRIS in the placebo arm. We found IL-6 (and IL-9 and PDGF) to be lower at week 2 in participants predicted to develop TB-IRIS receiving prednisone (Table 3.5 and Figure 3.4D).

TABLE 3.4 Concentrations of cytokines and chemokines at week 0 and week 2 in all participants by study arm

	Prednisone arm					Placebo arm					p (prednisone vs placebo)
	Week 0 (n = 86)		Week 2 (n = 79)		p	Week 0 (n = 86)		Week 2 (n = 72)		p	Week 2
IL-1b	0.12	(0.11-0.15)	0.12	(0.11-0.14)	0.98	0.12	(0.11-0.15)	0.13	(0.12-0.20)	0.02	0.01
IL-18	1083.5	(722.2-1670.6)	864.4	(524.1-1221.9)	***<0.0001***	1181.9	(841.5-1946.9)	972.6	(604.1-1993.3)	0.007	0.03
IL-1ra	107.0	(84.1-242.1)	98.5	(60.0-194.1)	0.05	127.7	(68.9-233.7)	218.5	(87.4-530.3)	0.004	***0.0001***
IL-6	0.10	(0.07-0.26)	0.10	(0.07-0.18)	0.32	0.10	(0.09-0.57)	0.24	(0.09-1.67)	0.02	***0.0002***
TNF	14.2	(10.3-21.5)	14.7	(8.2-30.2)	0.65	15.5	(10.5-24.4)	21.2	(11.1-36.0)	***0.0001***	0.007
IFN-y	0.38	(0.36-1.07)	0.36	(0.13-0.54)	***< 0.0001***	0.37	(0.33-0.87)	0.36	(0.20-0.62)	0.01	0.45
IL-12p70	0.33	(0.25-0.42)	0.33	(0.25-0.40)	0.30	0.34	(0.25-0.37)	0.36	(0.25-0.40)	0.08	0.40
IL-17	0.45	(0.34-0.50)	0.40	(0.32-0.46)	0.14	0.46	(0.34-0.61)	0.46	(0.34-0.78)	0.31	0.04
IL-2	1.10	(0.66-1.74)	1.11	(0.62-2.11)	0.76	1.11	(0.62-1.79)	1.26	(0.64-2.12)	0.01	0.24
IL-7	1.40	(1.14-1.80)	1.47	(1.40-2.01)	0.03	1.40	(1.14-2.01)	1.47	(1.26-1.90)	0.68	0.68
IL-4	0.15	(0.08-0.45)	0.14	(0.08-0.45)	0.44	0.16	(0.08-0.57)	0.16	(0.10-0.55)	0.002	0.45
IL-9	25.4	(20.9-32.4)	22.8	(17.9-29.9)	0.03	24.8	(21.1-32.0)	25.6	(21.3-30.5)	0.21	0.14
IL-13	0.05	(0.05-0.08)	0.05	(0.05-0.07)	0.05	0.05	(0.05-0.07)	0.05	(0.05-0.07)	0.81	0.55
IL-10	0.41	(0.31-0.63)	0.41	(0.31-0.58)	0.90	0.41	(0.28-0.50)	0.41	(0.31-0.70)	0.09	0.77
CCL2	13.3	(6.0-25.4)	8.84	(2.76-18.73)	***<0.0001***	16.5	(6.7-33.5)	8.86	(4.08-19.04)	***<0.0001***	0.59
CCL3	2.11	(1.22-3.74)	1.91	(1.11-5.12)	0.59	1.85	(1.21-3.28)	2.59	(1.66-6.33)	***0.0001***	0.04
CCL4	25.2	(20.9-32.9)	25.6	(20.5-33.8)	0.57	24.5	(20.4-29.7)	28.3	(23.2-36.9)	***0.0001***	0.04
CCL5	1469.9	(705.2-2726.1)	1066.3	(453.7-1687.3)	***<0.0001***	1207.2	(490.9-2662.6)	1176.1	(523.3-2141.5)	0.44	0.47
CCL11	9.15	(5.02-17.24)	8.46	(4.98-20.8)	0.40	7.96	(4.24-17.29)	8.05	(4.58-17.99)	0.005	0.79
CXCL8	1.36	(1.06-3.55)	1.06	(0.64-3.73)	0.41	1.16	(1.06-3.75)	1.57	(1.06-5.77)	0.03	0.11
CXCL10	907.3	(397.0-1561.0)	412.1	(219.3-983.1)	***0.0002***	872.7	(454.7-1680.1)	861.4	(389.8-1582.8)	0.83	***0.0003***
PDGF	23.0	(11.8-50.3)	18.8	(12.2-43.9)	0.24	20.5	(12.2-46.4)	27.2	(16.1-56.6)	0.03	0.19
basic FGF	2.07	(1.29-4.08)	1.94	(1.29-2.93)	0.18	2.02	(1.29-2.69)	1.97	(1.29-2.82)	0.48	0.77
CD14	4264.7	(3265.5-5401.8)	3908.0	(2825.9-5294.1)	0.10	4534.5	(3479.6-5658.1)	4782.5	(3653.7-5664.7)	0.50	0.01
CD163	1045.5	(809.2-1474.9)	1015.2	(724.3-1330.8)	0.39	1062.83	(820.1-1462.9)	1135.0	(751.9-1692.7)	0.06	0.08
TF	36.7	(30.0-46.9)	41.8	(31.2-52.1)	0.06	37.4	(31.7-45.4)	37.8	(30.7-45.5)	0.51	0.16

Data are shown as median (interquartile range). Concentrations (pg/ml) between participants in the prednisone and the placebo arm at week 2 were compared using the Wilcoxon rank sum test; concentrations between week 0 and week 2 were compared using the Wilcoxon matched-pairs signed ranks test (72 participants with in the prednisone arm vs 70 participants in the placebo arm). After adjusting for multiple comparisons using the Bonferroni correction, a p-value of < 0.002 was considered significant.

TABLE 3.5 Concentrations of cytokines and chemokines at week 2 in participants predicted and not predicted to develop TB-IRIS by study arm

	Predicted to develop TB-IRIS					Predicted to not develop TB-IRIS						
	Prednisone arm (n = 30)		Placebo arm (n = 33)		P1	Prednisone arm (n = 41)		Placebo arm (n = 37)		P2	P3	P 4
IL-1b	0.12	(0.09-0.14)	0.13	(0.11-0.2)	***0.04***	0.12	(0.11-0.14)	0.13	(0.12-0.19)	0.30	0.67	0.07
IL-18	947.0	(606.5-1286.5)	1333.5	(849.5-2318.9)	0.09	689.8	(420.8-1080.0)	753.8	(498.7-1333.2)	0.31	***0.02***	0.45
IL-1ra	120.6	(86.6-279.0)	338.2	(122.8-644.5)	***0.003***	80.0	(54.2-148.7)	139.4	(77.7-327.9)	***0.01***	***0.009***	0.80
IL-6	0.10	(0.07-0.18)	0.20	(0.09-0.54)	***0.01***	0.10	(0.09-0.18)	0.33	(0.10-1.96)	***0.007***	0.41	***0.002***
TNF	15.4	(8.8-27.4)	26.6	(18.7-37.8)	***0.007***	10.3	(7.7-31.8)	19.2	(10.1-29.5)	0.19	***0.02***	0.58
IFN-y	0.36	(0.2-0.67)	0.36	(0.19-0.64)	0.66	0.36	(0.11-0.36)	0.36	(0.29-0.60)	0.10	0.65	0.95
IL-12p70	0.31	(0.25-0.36)	0.33	(0.25-0.36)	0.28	0.36	(0.25-0.50)	0.36	(0.25-0.58)	0.86	0.25	0.11
IL-17	0.43	(0.34-0.56)	0.52	(0.34-1.01)	0.13	0.45	(0.32-0.46)	0.46	(0.34-0.58)	0.60	0.15	0.99
Il-2	1.24	(0.62-1.91)	1.44	(0.92-2.25)	0.15	1.06	(0.62-2.11)	1.22	(0.62-1.99)	0.82	0.21	0.92
IL-7	1.40	(1.14-2.01)	1.47	(1.40-1.80)	0.13	1.47	(1.40-2.11)	1.40	(1.03-2.10)	0.13	***0.03***	0.49
IL-4	0.19	(0.10-0.61)	0.18	(0.14-0.50)	0.92	0.14	(0.08-0.26)	0.14	(0.08-0.55)	0.52	0.59	0.50
IL-9	22.6	(17.6-28.2)	23.7	(21.3-28.3)	0.58	23.1	(20.6-30.9)	26.3	(22.3-33.4)	0.18	0.11	***0.04***
IL-13	0.05	(0.05-0.07)	0.05	(0.05-0.06)	0.30	0.05	(0.05-0.07)	0.05	(0.05-0.09)	0.64	0.96	0.68
IL-10	0.41	(0.31-0.81)	0.41	(0.31-1.00)	0.90	0.41	(0.31-0.42)	0.41	(0.27-0.63)	0.80	0.21	0.37
CCL2	10.6	(3.6-21.0)	9.29	(3.59-19.54)	0.66	7.43	(2.45-11.24)	8.89	(4.63-18.53)	0.28	0.96	0.89
CCL3	2.73	(1.41-7.27)	3.65	(2.03-8.11)	0.10	1.79	(1.14-4.94)	2.03	(1.26-3.26)	0.67	***0.004***	0.36
CCL4	26.5	(20.6-37.4)	29.1	(25.6-44.4)	0.10	26.2	(21.5-30.8)	27.3	(23.0-33.4)	0.41	0.11	0.50
CCL5	991.0	(452.6-1481.9)	1140.2	(470.4-2134.8)	0.62	1140.1	(684.1-1936.25)	1373.7	(576.4-2267.7)	0.64	0.40	0.17
CCL11	11.1	(5.8-20.8)	8.40	(4.55-18.59)	0.45	7.10	(4.04-15.55)	8.45	(4.60-17.8)	0.67	0.99	0.35
CXCL8	1.57	(0.64-3.73)	1.94	(1.06-8.85)	0.16	1.06	(0.61-2.22)	1.32	(0.98-5.52)	0.50	0.15	0.99
CXCL10	455.3	(202.4-1052.3)	1166.1	(598.9-1928.6)	***0.003***	412.1	(230.8-827.4)	660.0	(281.7-1353.3)	0.09	0.05	0.16
PDGF	18.6	(11.8-40.9)	24.3	(16.2-39.2)	0.52	22.7	(13.5-51.4)	32.8	(16.2-73.6)	0.24	0.07	***0.04***
basic FGF	1.97	(1.29-2.93)	2.02	(1.29-2.93)	0.88	2.02	(1.29-2.96)	1.82	(1.29-2.77)	0.78	0.61	0.82
CD14	4001.4	(3118.3-5921.1)	5166.1	(4367.5-5856.4)	0.08	3894.4	(2789.2-5056.4)	4160.3	(3028.7-5272.8)	0.23	***0.02***	0.94
CD163	1148.5	(865.0-1749.7)	1170.5	(719.5-1780.1)	0.91	868.7	(679.3-1226.4)	1085.7	(858.4-1509.8)	0.05	0.72	0.58
TF	42.3	(35.2-55.1)	38.2	(30.0-45.0)	0.06	37.6	(30.8-45.9)	39.0	(31.0-47.9)	0.88	0.64	0.07

Data are shown as median (interquartile range). Concentrations were compared using the Wilcoxon rank sum test. P1 relates to the comparison of chemo- and cytokines between the prednisone and placebo arm in participants predicted by the model to develop TB-IRIS; P2 relates to with the comparison of chemo- and cytokines between the prednisone and the placebo arm in participants predicted by the model to not develop TB-IRIS; P3 relates to with the comparison of chemo- and cytokines between participants predicted to develop and not to develop TB-IRIS in the placebo arm; P4 relates to with the comparison of chemo- and cytokines between participants predicted by the model to develop TB-IRIS who received prednisone and participants predicted by the model to not develop TB-IRIS who received placebo. A p-value of < 0.05 was considered significant.

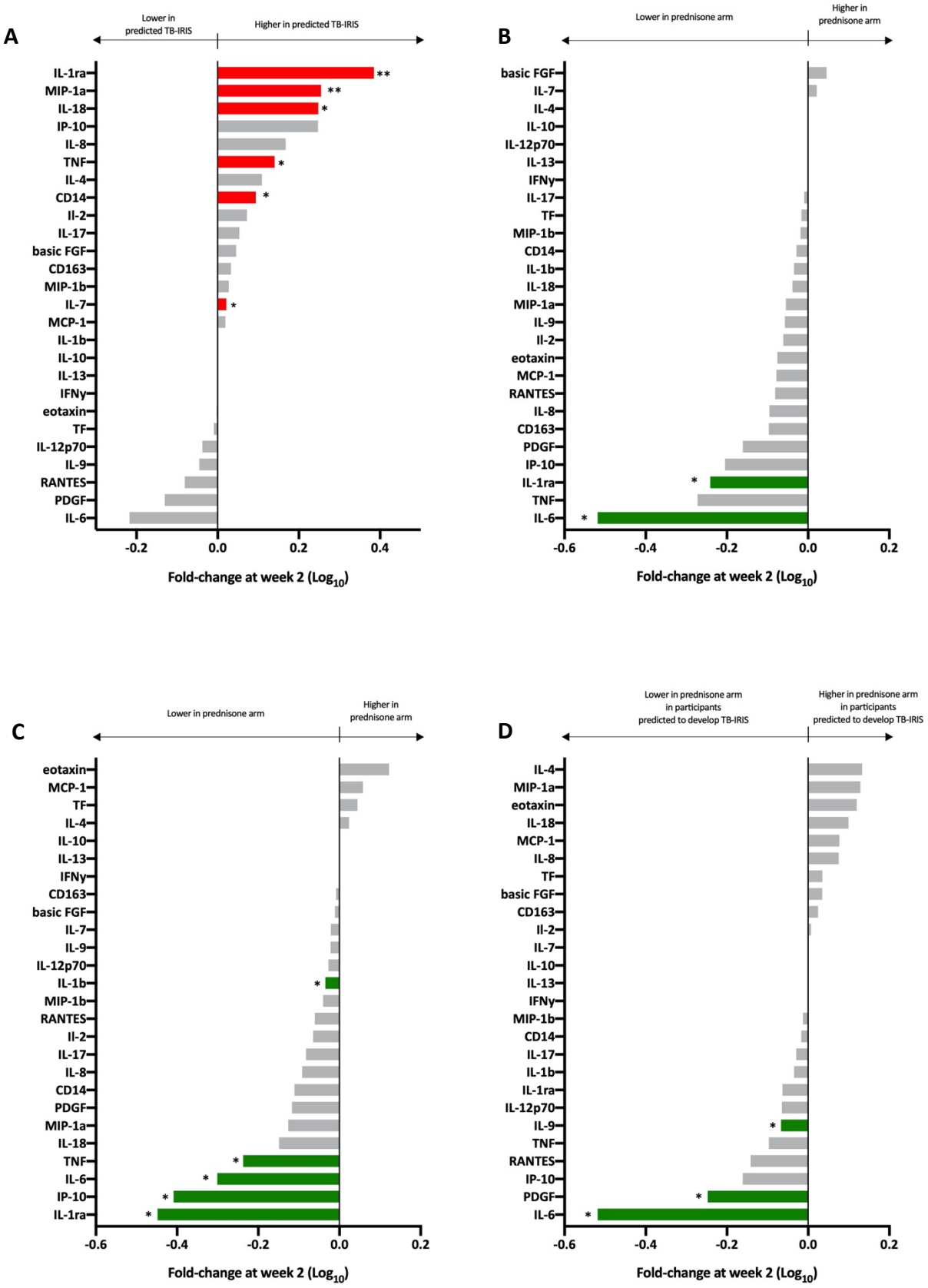

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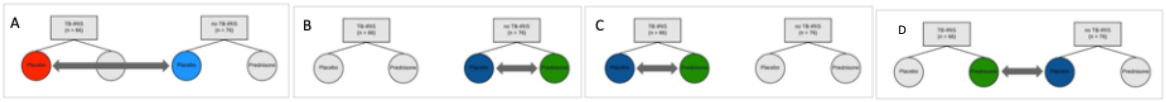
FIGURE 3.4 Fold-change of concentrations of cytokines and chemokines at week 2 comparing different groups of participants from the model predicting the development of TB-IRIS.

Concentrations were compared between participants using the Wilcoxon rank sum test. A p-value of < 0.05 (*) was considered significant.
A comparison between participants predicted to develop and not to develop TB-IRIS in the placebo arm**; B** comparison between the prednisone and the placebo arm in participants predicted not to develop TB-IRIS; **C** comparison between the prednisone and placebo arm in participants predicted to develop TB-IRIS; **D** comparison between participants predicted to develop TB-IRIS who received prednisone and participants predicted to not develop TB-IRIS who received placebo. Comparisons (refer to figure 3.3A) are depicted below:



No association between LTA4H genotype and the development of TB-IRIS was demonstrated.

Among the 213 participants who had LTA4H genotyping performed, 173 (81%) had a CC, 31 (15%) had a CT and 9 (4%) had a TT genotype. The overall observed distribution of alleles roughly resembles the African distribution (Ensembl.org) (Figure 3.5), however our findings were not consistent with the Hardy Weinberg equilibrium (p < 0.0001).

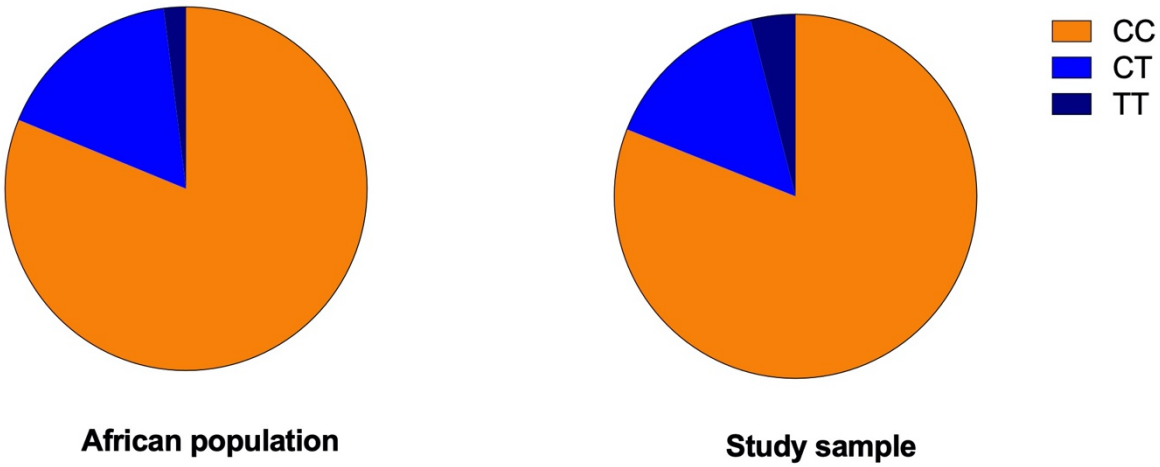


FIGURE 3.5 Distribution of *lta4h* genotypes in the African population and our study sample.

Because of the low frequency of mutant genotypes CT and TT, we grouped them together for further analysis. We found no association between LTA4H genotype and the development of TB-IRIS, both in the entire cohort (p = 0.88) as well as in participants in the placebo arm only (p = 0.51) (Figure 3.6).

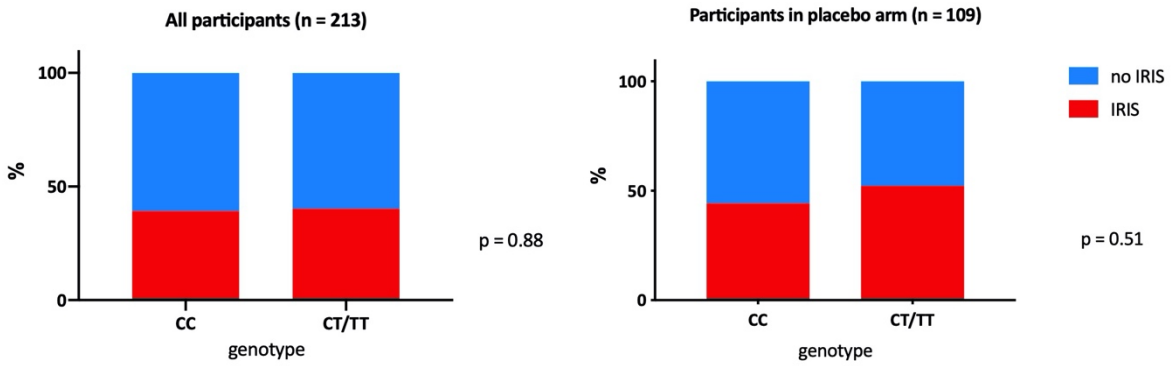


FIGURE 3.6 Association between *lta4h* genotype and TB-IRIS

Comparisons were done using the Pearson Chi square test. A p-value of < 0.05 was considered significant.

No association between genotype and cytokine/chemokine concentrations at baseline or two weeks after start of ART was demonstrated

We compared different chemo- and cytokines between participants with a wildtype and a mutant LTA4H genotype. We found no association between genotype and cytokine profile, at week 0 or at week 2 (Table 3.6).

TABLE 3.6 Cytokine concentrations comparing wildtype (CC) vs mutant (CT and TT) *lta4h* genotype at week 0 (start ART) and week 2.

	Week 0					Week 2				
	Wildtype (CC) (n = 124)		Mutant (CT/TT) (n = 32)		p-value	Wildtype (CC) (n = 105)		Mutant (CT/TT) (n = 28)		p-value
IL-1b	0.12	(0.11-0.14)	0.14	(0.12-0.18)	0.03	0.12	(0.11-0.16)	0.15	(0.11-0.2)	0.16
IL-18	1125.8	(793.2-1946.9)	901.0	(647.9-1629.1)	0.14	890.1	(564.7-1333.2)	894.6	(506.5-1493.3)	0.72
IL-1ra	112.7	(73.7-235.4)	116.8	(91.2-267.3)	0.60	122.8	(73.5-299.8)	157.5	(90.0-520.6)	0.28
IL-6	0.10	(0.09-0.31)	0.10	(0.07-0.24)	0.66	0.14	(0.09-0.61)	0.12	(0.09-0.42)	0.85
TNF	14.1	(9.7-22.9)	18.5	(14.0-25.1)	0.03	17.9	(9.1-32.4)	20.2	(9.8-30.6)	0.39
IFN-y	0.36	(0.32-0.86)	0.39	(0.34-0.90)	0.73	0.36	(0.19-0.55)	0.36	(0.24-0.65)	0.33
IL-12p70	0.33	(0.25-0.41)	0.34	(0.25-0.36)	0.91	0.34	(0.25-0.40)	0.33	(0.25-0.36)	0.44
IL-17	0.46	(0.34-0.51)	0.34	(0.25-0.55)	0.36	0.46	(0.34-0.58)	0.46	(0.28-0.79)	0.99
IL-2	1.07	(0.62-1.59)	1.28	(0.79-2.27)	0.17	1.22	(0.62-2.11)	1.06	(0.63-2.39)	0.95
IL-7	1.40	(1.14-1.64)	1.47	(1.14-2.48)	0.33	1.40	(1.14-1.80)	1.47	(1.40-2.39)	0.10
IL-4	0.15	(0.08-0.54)	0.18	(0.08-0.46)	0.95	0.14	(0.08-0.51)	0.22	(0.14-0.46)	0.24
IL-9	24.4	(19.5-32.5)	28.3	(22.1-31.4)	0.26	23.7	(19.4-30.4)	26.1	(23.2-30.3)	0.14
IL-13	0.05	(0.05-0.07)	0.05	(0.05-0.06)	0.32	0.05	(0.05-0.07)	0.05	(0.05-0.06)	0.40
IL-10	0.41	(0.31-0.58)	0.41	(0.31-0.54)	0.84	0.41	(0.31-0.58)	0.41	(0.31-0.95)	0.48
CCL2	12.4	(5.8-25.8)	16.5	(6.9-26.1)	0.46	8.14	(2.70-17.81)	9.82	(5.42-20.25)	0.15
CCL3	1.92	(1.15-3.35)	2.00	(1.12-3.89)	0.88	2.25	(1.39-5.63)	2.15	(1.10-6.36)	0.67
CCL4	24.4	(19.4-30.0)	25.5	(21.6-31.2)	0.27	26.1	(21.5-33.9)	28.8	(23.9-39.4)	0.18
CCL5	1289.6	(572.5-2764.4)	1178.8	(477.7-2692.1)	0.68	1208.2	(546.7-1953.0)	1082.6	(496.5-2414.1)	0.94
CCL11	9.43	(4.45-18.14)	8.62	(5.29-16.35)	0.94	8.35	(4.38-20.8)	11.2	(6.0-17.4)	0.55
CXCL8	1.21	(0.91-3.64)	1.32	(1.06-4.25)	0.50	1.40	(0.91-4.61)	1.09	(1.02-3.98)	0.84
CXCL10	997.2	(406.0-1595.3)	848.1	(552.9-1636.4)	0.86	602.5	(281.7-1326.9)	675.4	(361.6-1406.0)	0.59
PDGF	20.1	(11.9-50.4)	21.3	(11.5-37.4)	0.63	22.6	(13.4-52.7)	30.4	(15.7-57.8)	0.31
basic FGF	1.95	(1.29-3.31)	2.04	(1.29-2.85)	0.79	1.94	(1.29-2.93)	2.02	(1.29-2.70)	0.71
CD14	4285.8	(3347.3-5401.3)	4404.7	(3397.3-5772.0)	0.61	4162.0	(3028.7-5294.1)	4195.2	(3314.1-5531.0)	0.82
CD163	1063.3	(822.0-1462.9)	1015.9	(711.7-1474.9)	0.54	1039.6	(727.6-1491.8)	986.1	(727.6-1296.6)	0.77
TF	37.3	(30.8-43.6)	34.9	(29.5-46.2)	0.56	38.7	(30.7-45.9)	37.6	(31.1-49.2)	0.69

Data are shown as median (interquartile range). Concentrations were compared using the Wilcoxon rank sum test, adjusting for multiple comparisons using the Bonferroni correction. A p-value of < 0.002 is considered significant.

TABLE 3.7 The effect of LTA4H genotype on the efficacy of prophylactic prednisone

	Hazard ratio	95% CI	p-value
Prednisone arm	0.66	0.41 – 1.07	0.10
Mutant genotype	1.14	0.59 – 2.24	0.69
Interaction	0.66	0.21 – 2.11	0.49

Cox proportional hazard models with time to TB-IRIS as outcome and treatment arm, genotype and their interaction as variables.

There was no association shown between LTA4H genotype and the efficacy of prednisone to prevent TB-IRIS

We assessed whether LTA4H genotype affects the efficacy of prednisone to prevent TB-IRIS. Although prednisone seems to prevent TB-IRIS more effectively in the mutant genotype group (HR 0.66, 95% CI 0.21 – 2.11), the difference was not statistically significant (p = 0.49) and a wide confidence interval prevented us from making any conclusions regarding efficacy in relation to genotype (Table 3.7 and Figure 3.7).

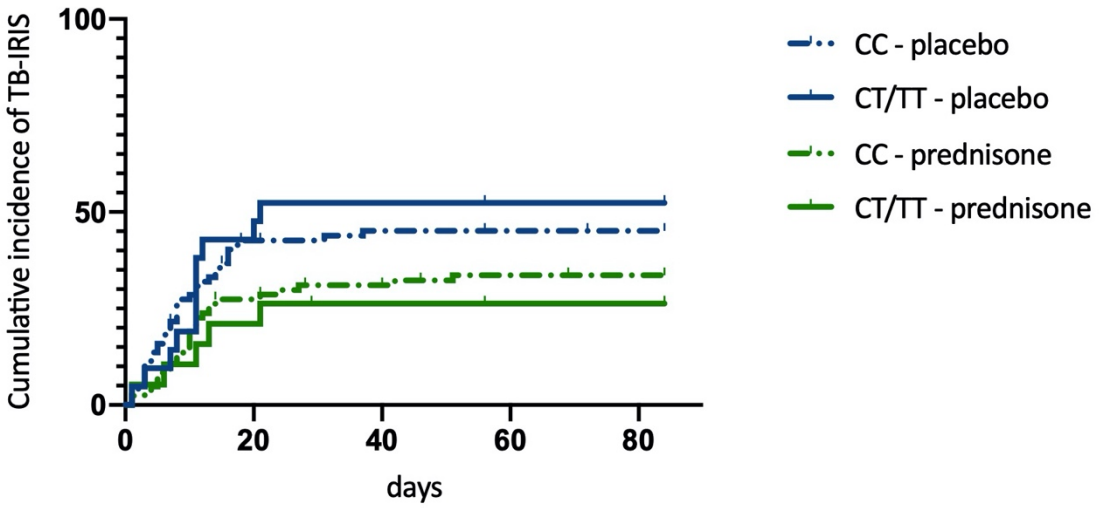


FIGURE 3.7 Cumulative incidence of TB-IRIS by *lta4h* genotype and study arm.

Discussion

In our study, we have assessed the association between soluble chemo- and cytokines and TB-IRIS in a cohort of participants at high risk of TB-IRIS receiving either prednisone or placebo from the initiation of ART.

We found no statistically significant association between plasma concentrations of chemo- or cytokines at baseline and the development of TB-IRIS. Several other studies have evaluated chemo- and cytokine concentrations to predict TB-IRIS. Although individual studies describe roles for certain cytokines in predicting TB-IRIS, assessing studies together shows that baseline concentrations of individual cytokines are generally not associated with TB-IRIS across studies [21, 38, 48, 60-66, 146, 147], a finding we confirmed in our study. Our finding needs to be interpreted with caution, however, as participants in our cohort have been selected for their high risk of TB-IRIS and may therefore be more uniform than a less selected population.

As described in other studies [59, 67], we found TB-IRIS was associated with increased concentrations of several chemo- and cytokines 2 weeks after starting ART. Particularly TNF-α, CXCL-10, CCL-3, IL-1ra and IL-2 were increased, with a trend for higher IL-1 β, IL-6 and most of the other chemokines. TNF-α has long been associated with both protection and pathology in TB [86] and raised plasma concentrations have been found in patients with TB-IRIS [21, 59, 61-63, 67], a finding we confirm in our study. CXCL-10 plasma concentrations are raised in patients with active TB with and without HIV [148] and decline during TB treatment [149, 150]. Similar to other studies [60, 62, 66], we found raised concentrations of plasma CXCL-10 in participants with TB-IRIS when compared to non TB-IRIS controls, it appears because concentrations did not decline on ART in patients with TB-IRIS [62, 66]. However, not all studies show these increased plasma CXCL-10 concentrations during TB-IRIS [61, 63, 151], possibly as a result of difference in time between starting antituberculous therapy and ART between the TB-IRIS patients and non-TB-IRIS controls. Our finding of increased concentrations of CCL-3 in participants with TB-IRIS is not reported consistently in the literature, with both increased and decreased CCL-3 concentrations found during both TB and TB-IRIS [61, 63, 152-155]. We found increased concentrations of IL-1ra in participants with TB-IRIS. IL-1ra is a naturally occurring competitive inhibitor of the pro-inflammatory cytokines IL-1α and IL-1β. Increased plasma concentrations have been found in patients with TB [156, 157] and TB-IRIS [61, 64, 65]; the latter may represent compensatory anti-inflammatory and immune-regulatory responses accompanying the excess inflammation of TB-IRIS. IL-1ra is secreted by monocytes together with IL-1β. Because of the short half-life time of IL-1β, making it difficult to measure, IL-1ra has been used as a surrogate for IL-1β [65]. So alternatively, rather than reflecting decreased inflammation, increased IL1-ra concentrations in our study may mirror increased IL-1β concentrations.

Initial studies of IRIS showed an increase in antigen-specific, IFN-γ producing CD4+ T cells and their pro-inflammatory cytokines associated with IRIS [46, 52]. Subsequent studies showed increases in polyfunctional (co-expressing IFN-γ, TNF-α and IL-2) tuberculosis-specific CD4+ T-cell responses were associated with TB-IRIS [49, 50]; these are considered an effector memory population [48, 51]. These cells may simultaneously stimulate innate (macrophage activation via IFN-γ and TNF-α) and adaptive (T cell proliferation via IL-2) immune responses. More data emerged pointing to a role for the innate immune system in the pathogenesis of TB-IRIS [38, 60, 64, 67]: TB-IRIS associates with increased concentrations of IL-6 and IL-18; and a transcriptomic signature of TB-IRIS predicts activation of innate signalling pathways and production of proinflammatory cytokines and chemokines [67]. Moreover, monocytes and macrophages have been associated with TB-IRIS. The role of macrophages in TB-IRIS was first suggested in a case report of unmasking TB-IRIS describing macrophages as the dominant cell type present in affected lung tissue [158]. An animal model of TB-IRIS, using T-cell deficient mice, showed similar findings [159]. Subsequent studies further suggest a role for monocytes or macrophages in TB-IRIS [160], especially expansion and activation of the $CD14^{++}$ $CD16^{-}$ subset [61].
Our data support a conclusion that an interaction between the innate and adaptive immune responses plays a role in TB-IRIS. The increased concentrations at the time of TB-IRIS of IL-2 and CXCL-10 indicate the involvement of a Th1 response, whereas increased concentrations of TNF-α and CCL-3 could indicate macrophage activation. However, we did not find increased concentrations of sCD14 and sCD163 - markers associated with monocyte and macrophage activation. This could of course mean factors other than macrophage activation are involved in the increased concentrations of TNF-α and CCL-3.

Prophylactic prednisone, when compared to placebo, was associated with lower plasma concentrations at week 2 of those chemo- and cytokines that were found to be increased in TB-IRIS – with the exception of IL-2. This is in line with previous studies showing a decrease in plasma concentrations of several cytokines (IL-6, IL-10, IL-12p40, IFN-γ, CXCL-10, and TNF-α) in patients treated with prednisone for TB-IRIS [139] or pericardial tuberculosis [161] compared to placebo. Because prednisone reduces the incidence of TB-IRIS [43] it is not possible to conclude from our study whether lower concentrations in the prednisone arm mediated the reduction or are a consequence thereof. Moreover, discerning the effect of prednisone could be diluted when given to all participants and not only to participants who will develop TB-IRIS. We tried to overcome these limitations by developing a model that predicted which participants would develop TB-IRIS and which would not, repeating the analysis only including participants predicted to develop TB-IRIS. We reasoned that if the subgroup of these participants allocated to the prednisone arm subsequently did not develop TB-IRIS, this could be attributed to prednisone. Comparing prednisone to placebo in participants predicted to develop TB-IRIS, we could confirm lower concentrations of IL-1ra, TNF-α and CXCL-10 as well as IL-1β and IL-6 in participants in the prednisone arm. We further reasoned that if prednisone modifies the cytokine profile of participants predicted

to develop TB-IRIS to resemble the cytokine profile of participants without TB-IRIS, their cytokine profile at week 2 would resemble the cytokine profile of participants not predicted to develop TB-IRIS in the placebo arm. We identified cytokines with lower plasma concentrations in the prednisone arm compared to the placebo arm in participants predicted to develop TB-IRIS. We compared their concentrations to cytokine concentrations of participants in the placebo arm of participants predicted to *not* develop TB-IRIS, and found the difference in concentrations was no longer statistically significant. The only exception is IL-6, which is still lower in the prednisone arm. However, because of small numbers in all compared groups, it is not possible to draw solid conclusions.

Corticosteroids inhibit inflammatory signals via three different mechanisms: regulation of gene expression via interaction with glucocorticoid-responsive element; interaction with transcription factors such as NF-κB – occurring at lower corticosteroid concentrations; and signaling through membrane-associated receptors and second messengers [162]. NF-κB has also been proposed to play a role in TB-IRIS, triggering the production of an array of cytokines, including TNF-α and IL-6 or activating the inflammasome, leading to the production of IL-1β [163].

We assessed *lta4h* genotype in patients at risk for TB-IRIS. We found our study sample did not comply with the Hardy Weinberg equilibrium. An explanation for the lower than expected proportion of heterozygotes in our cohort could be the large proportion of participants with extrapulmonary TB; in some studies the heterozygote CT genotype was associated with a decreased risk for extrapulmonary TB [96, 141] - although this association has not been found in other studies [140-142].

LTA4H directly affects LTB4, a strong attractor of neutrophils and macrophages to sites of inflammation. Therefore, one could expect *lta4h* genotype to both associate with inflammation in TB-IRIS and influence the plasma chemo- and cytokine profile. However, we did not find an association between *lta4h* genotype and TB-IRIS, similar to the only other study to date assessing LTA4H and TB-IRIS [99]. We also did not find an association between *lta4h* genotype and cytokine profile. Only one other study assessed this association, in cerebrospinal fluid (CSF) rather than in plasma, in patients with tuberculous meningitis (TBM). The authors found higher concentrations of IL-1β, IL-2 and IL-6 in patients with mutant genotypes (CT and TT), but only in those not infected with HIV [144]. Possible reasons why we did not find this association between genotype and cytokine concentration include: (1) A possible difference in the role of LTA4H in HIV-infected and uninfected patients: in the above-mentioned study *lta4h* genotype did not affect CSF cytokine concentrations in HIV-infected patients, and a survival benefit for those with a TT genotype was also only evident for patients not infected with HIV [144]. Moreover, HIV affects the ability of neutrophils [164] and alveolar macrophages [165] to produce LTB4 in vitro; LTB4 - usually elevated in bronchoalveolar lavage (BAL) fluid of patients with bacterial pneumonia

[166] - was not elevated in HIV infected patients with pneumonia compared to healthy controls [167]. (2) The difference between pulmonary TB and TBM: studies showing a difference in clinical picture and genotype all relate to TBM [96, 141], whereas studies assessing pulmonary TB did not find this association [140-142]. LTA4H is not only involved in generation of the pro-inflammatory LTB4, it also breaks down Proline-Glycine-Proline (PGP) [168]. PGP is a tripeptide that is generated from collagen by matrix metalloproteinases upregulated in TB. It attracts neutrophils and plays a role in inflammatory lung disease [169, 170]. Although our study cohort included many participants with not only pulmonary but also extrapulmonary TB, patients with TBM were excluded from the trial. If the role of LTA4H as anti-inflammatory enzyme – by breaking down PGP - is more important in pulmonary TB, whereas its role as pro-inflammatory enzyme is more important in TBM, this might explain our findings.

We did not find a statistically significant effect of *lta4h* genotype on the efficacy of prednisone to prevent TB-IRIS. This could be due to the low frequency of mutant alleles in our African study population (10%) compared to other studies performed in Southeast Asia, where mutant alleles are much more frequent (33%) (Ensembl.org). However, a study done in South India, in which 40% of the participants had a mutant genotype, also did not find the response to steroids (used as treatment for TB-IRIS, not as prophylaxis) to be genotype dependent [99].

We recognize an important limitation to our study. Because inclusion of participants in our trial took place over a period of 2.5 years, there is a difference in storage time between the samples. Most cytokines are stable when stored at -80 degrees for a period of 2 years but can degrade afterwards. In a study assessing the effect of long term storage on cytokine concentrations, concentrations of IL-1β IL-5, IL-8, IL-10, IL-13 and IL-15 all decreased below 50% after 4 years [171]. Our samples have been stored for a median of 3.5 years. We recognize that as a consequence, we might not be able to measure these unstable cytokines. We calculated median storage time, which did not differ between the different groups. Assuming that cytokine degradation over time occurs with similar kinetics in all groups, we can still reliably interpret differences in cytokine concentrations between groups. We have chosen to assess chemo- and cytokines measuring direct plasma levels rather than using cell stimulation assays because of convenience, the ease of performance, and a relatively low cost. We realize that would we have used cell stimulation assays, levels of chemo- and cytokines may have been less affected by short half-life times of individual analytes as well as degradation over time. Moreover, simultaneously assessing changes in the frequency and immunophenotype of relevant circulating immune cells like monocytes or T-cells, rather than only analyzing plasma analytes, would have been able to better guide us towards the origin of the various chemo- and cytokines.

Due to a relatively small sample size, we are limited in our ability to assess the effect of TB-IRIS or prednisone in certain subgroups (placebo arm only or participants predicted to develop TB-IRIS only. Moreover, a difference in chemo- and cytokine levels between groups may have been detected if we had been able to include more participants in our analysis.

In conclusion, our findings support previous findings that TB-IRIS is characterized by excess inflammation and hypercytokinemia, with a prominent role for the innate immune system. Prednisone used to prevent TB-IRIS was associated with a decrease in these cytokines. In our study, l*ta4h* genotype associated with neither the incidence nor the cytokine profile of TB-IRIS. We were unable to show that the efficacy of prednisone to prevent TB-IRIS is genotype dependent.

The immune mechanisms of lung parenchymal damage in tuberculosis and the role of host-directed therapy

Introduction

In 2016, an estimated 10.4 million people developed tuberculosis (TB) worldwide. Although effective diagnosis and treatment saved about 53 million lives between 2000 and 2016, TB remains a major threat worldwide: 16% of TB cases die from the disease, corresponding to 1.7 million deaths in 2016 [172]. Among those who are cured successfully, residual pulmonary impairment is common. Various studies have looked at lung function in patients with a known history of TB; they found abnormal lung function in 34% - 94% of patients, varying in severity from mild to severe [112, 114, 116, 124, 173-179]. It results in considerable medical costs [180] and decreased quality of life [124, 175].
Impaired lung function is associated with chest radiograph (CXR) abnormalities in most of the studies. It can easily be measured using spirometry, which measures air volumes and airflow rates of the lung. Forced vital capacity (FVC) is the maximal volume of air exhaled by a patient from the position of maximal inspiration, by means of a rapid, maximally forced expiration; forced expiratory volume in 1 second (FEV1) is the amount of air exhaled during the first second of the FVC maneuver. The nature and severity of pulmonary impairment can be categorized by combining these two measurements: obstruction is defined as a FEV1 / FVC ratio < 70%, restriction is suggested by a low FVC (< 80% of the predicted value). Obstruction, low FVC, and mixed defects have all been reported in patients with previous TB.

Purpose of review

The aim of TB treatment is to kill the causative mycobacteria with anti-mycobacterial agents. Because of the lengthy duration of the treatment, the possibilities of drug toxicity, and increasing drug resistance, host-directed therapies (HDT), have gained attention [92, 181, 182]. HDTs are agents that can augment host defense mechanisms, modulate excessive inflammation or both, by manipulating the hosts response to a pathogen rather than targeting the pathogen itself. This may lead to improved clinical treatment outcomes such as reduced morbidity, mortality, and end-organ damage, and long-term functional recovery. Supplementing anti-TB treatment with drugs that reduce pulmonary damage could result in improved pulmonary function. To predict which interventions could be beneficial, an

understanding of the pathogenesis of pulmonary damage in TB is important. What are the immunological processes leading to lung damage in humans? Where and how in the process could we intervene to prevent or reduce lung damage? How much damage is already done at diagnosis and how much still occurs during treatment?

What does pulmonary damage in human TB look like?

The established paradigm positions the caseating granuloma as the characteristic lesion of TB. However, this paradigm originates from animal studies in the late 20th century, when data on histology of human TB had become rare. Studies done before the 1950s describe two characteristic presentations in human pulmonary TB: the caseous granuloma and the tuberculous pneumonia. They divide lung pathology into primary and post-primary TB. Primary TB is the infection that occurs when people first encounter *Mycobacterium tuberculosis* (*Mtb*). Post-primary TB occurs later, as a result of reactivation of latent TB or reinfection, and causes the majority of clinical TB [183]. The two differ with regard to their location in the lung, the host immune response and their histopathology. Primary TB typically occurs mainly in the lower zones of the lung. It is usually self-limiting but leads to consolidative pneumonia or lymphadenitis in a small proportion of individuals. It is characterized by a greater bacillary load and reduced lipid accumulation in the alveoli and the interstitium compared to post-primary TB, as well as an acute inflammatory response; cavitation however, is rare. Post-primary TB is said to develop mainly in the apices of the lung. It is characterized by obstructive pneumonia, which is frequently asymptomatic in its early stages. Endobronchial spread from the small peripheral airways can lead to necrotic caseous pneumonia, associated with progressive tissue necrosis and cavity formation or fibrocaseous disease [184, 185]. TB typically heals with persisting cavities, scarring, and pleural adhesions, as observed in autopsies of persons with previous TB who died of other causes [186]. However, abnormal findings need not be present and viable TB can be found in both macroscopically normal and abnormal appearing lung tissue [187].

Chest radiographs (CXR) are commonly used to visualize pulmonary damage. Radiologists distinguish primary and post-primary TB as the two typical patterns in active TB. Primary TB is characterized by lymphadenopathy and air space consolidation often in the middle or lower lobes, with or without an accompanying pleural effusion. Post-primary TB consists of consolidation and/or nodules, frequently in the upper lobes or apices of the lower lobes, with or without cavitation [188]. CXRs of people with previous TB show abnormalities in 14-100%, including fibrosis, bronchiectasis, and persisting cavities, the latter occurring more often in re-treatment patients or those with multi-drug resistant TB [189]. All these abnormalities are associated with impaired lung function.

Computed tomography (CT) scans are more sensitive than CXRs, especially for imaging of centrilobular small nodules or the so-called tree-in-bud sign; these classical features of early endobronchial spread of TB are often underestimated on a CXR [190];
[18F]-fluoro-2-deoxy-D-glucose positron emission tomography (FDG-PET) with CT combines anatomic imaging with imaging of metabolic activity of lesions. It has been used in TB to follow the evolution of lung lesions during treatment [191, 192] and, importantly, has shown that metabolically active lung lesions may be present before the onset of clinical disease [193], and persist after treatment completion [191].

What happens after *Mtb* enters the lung?

After *Mtb* enters the lung, the bacilli are taken up by alveolar macrophages, dendritic cells, and neutrophils, or occasionally epithelial cells; the latter possibly resulting in limited early bacterial growth. Infected cells start producing and secreting antimicrobial peptides, cytokines (like interleukin (IL)-1β, tumor necrosis factor (TNF)-α, IL-12, and IL-6) and chemokines. Other immune cells and permissive macrophages are attracted to the site of infection [86]. *Mtb* itself, using multiple strategies, directs the recruitment of macrophages and triggers granuloma formation [194]. Secondary granulomas are formed by infected macrophages departing the primary granuloma or when a granuloma ruptures. While *Mtb* replicates freely in the macrophages, dendritic cells migrate to the local lymph nodes, to activate T cells. The arrival of *Mtb* specific T-cells in the lung usually does not happen until 14-21 days after initiation of the infection [195]. Their production of TNF-α and interferon-γ (IFN-γ) stimulates killing activities by macrophages. Moreover, T-cells complete granuloma formation by forming the lymphocytic cuff surrounding it [86].
The balance between the eicosanoids prostaglandin E2 (PGE2) and lipoxin A4 (LXA4) affects the mode of death of infected macrophages. LXA4 promotes macrophage necrosis, resulting in cell lysis of the macrophage, thereby allowing *Mtb* to escape and spread to neighboring cells. PGE2 stimulates apoptosis, leaving the macrophage plasma membrane intact, containing the bacilli, and enhancing immunity [196]. Leukotriene (LT) B4, through regulation of TNF-α production [96] and possibly attraction of neutrophils [197], is also involved, with both high and low levels of LTB4 inducing macrophage necrosis [96].
In only 10% of individuals, progressive primary disease occurs; in the remaining 90% the initial infection is contained and latent infection is established [86]. Current thinking views active and latent TB on a spectrum of tuberculosis disease, rather than as two distinct disease states as historically classified. [198].

Granulomas

Most human granulomas are composed of a center of infected macrophages, with the ability to differentiate, for example into epithelioid cells, multi-nucleated giant cells, and foamy macrophages. An outer layer of lymphocytes surrounds these cells, and many other cells, including neutrophils, dendritic cells, natural killer (NK) cells and fibroblasts may form part of the granuloma. The granuloma contains the mycobacteria, preventing their spread, but at the same time serves as a site of replication and persistence for *Mtb* [194]. Different types of granuloma exist: cellular, suppurative, fibrotic, or caseous [199]. Caseous necrosis occurs when cells within the granuloma undergo necrosis [86]; alternatively, it has been suggested that - in post-primary TB - granulomas form in response to existing areas of necrotic caseous pneumonia [184]. Caseous necrosis happens in conjunction with extracellular matrix (ECM) destruction. In the classical paradigm, tissue destruction occurs as a result of caseous necrosis [86]. However, an alternative theory proposes that collagen destruction precedes caseation and, therefore, ECM destruction is the initial pathological event [200].

Diverse types of granulomas can be present in one lung at the same time, ranging from small cellular granulomas to multiple caseous granulomas that coalesce and expel their contents to form large cavities; they behave independently of each other, and different immunologic profiles exist between [201, 202] and within [203] granulomas. Granulomas can be stable, or either resolve or progress. Clinically, the behavior of a few or even a single poorly controlled granuloma can determine the outcome of the disease on a host level [204].

Cavities, bronchiectasis and fibrosis

The lung consists of both cellular and extracellular components. The ECM is comprised of the interstitial connective tissue matrix, which forms the parenchyma of the lung, surrounding cells and providing structural scaffolding, and the basement membrane, which separates the alveolar epithelium or endothelium from the surrounding stroma. Support of the alveoli by the ECM is needed for normal lung function; destruction or abnormal remodeling of the ECM occurs in many pulmonary diseases and leads to pulmonary impairment [205]. The ECM of the lung is mainly made up of type I collagen and elastin. Type III and IV collagen are important components of the alveolar wall and basement membrane. Large fibers are connected by smaller fibrils. Dissemination of mycobacteria from the lung parenchyma into the airways as well as formation of cavities requires destruction of the ECM through cleavage of both small fibrils and large fibers. Collagens, however, are highly resistant to cleavage by proteolytic enzymes; only matrix metalloproteinases (MMPs) are capable of completely degrading the ECM [205].

Consequently, MMPs play an important role in the development of cavities, bronchiectasis as well as fibrosis.
The development of cavities in TB has been studied extensively in rabbits, using Mycobacterium bovis. In these studies, cavities developed from liquefied caseating granulomas, that contained large numbers of actively growing bacteria. Bacteria release high amounts of tuberculin-like products causing a tissue-damaging delayed-type hypersensitivity reaction [206]. This T-cell mediated immune reaction is important; cavities developed mainly in pre-sensitized rabbits and desensitization or immune suppression could prevent cavity formation [207, 208]. Cavities are formed when expanding granulomas ruptures their caseous contents into a bronchus [206].
Histologic studies in humans show a different picture of cavity formation that challenges the paradigm described in rabbits [184]: cavities do not develop from liquefied caseating granulomas, but from a caseous pneumonia. Host lipids and mycobacterial antigens accumulate in the alveoli, but only small numbers of bacteria are present. Similar to the rabbit model, sudden necrosis related to a delayed-type hypersensitivity reaction against mycobacterial antigens occurs [184]. However, an alternative yet controversial theory, based on the small numbers of bacteria observed and several observations related to autoimmunity seen in patients with TB, proposes a role for autoimmunity: mycobacteria induce inappropriate host responses to self-antigens, causing autoimmune inflammation [209]. A considerable overlap in gene expression signatures between TB and autoimmune diseases, greater than seen with other infectious diseases, supports this theory [210].
The lipid-rich necrotic material in granulomas does not have the enzymatic activity to degrade collagen and consequently, its build-up is only one component of cavity formation. Extracellular matrix breakdown takes place and involves MMPs. Indeed, increased concentrations of MMPs have been found in TB cavities in rabbits [211] and in humans [212, 213]. Neutrophils have also been found in cavities [212].

Bronchiectasis, an irreversible dilatation of the bronchi, is caused by an ongoing inflammatory process (like TB), which results in damage to the airway epithelium, leading to an inability to clear secretions, as well as destruction of the elastin in the airway walls [214]. Similar to cavity formation, MMPs have been implicated in the development of bronchiectasis, with increased levels being found in sputum, bronchoalveolar lavage fluid (BALF), and the lamina propria of patients with bronchiectasis [215-217]. Neutrophils, together with macrophages and T-cells, are the dominant cell type in bronchiectatic inflammation [218]. Alternatively, traction bronchiectasis can occur, secondary to scarring of the adjacent parenchyma or narrowing of more proximal bronchi [214].

Fibrosis results from the excessive deposition of components of the ECM such as collagen and fibronectin in and around inflamed or damaged tissue by myofibroblasts. Its pathogenesis is complicated [219], with many innate and adaptive immune cells and cytokines playing a role. Transforming growth factor (TGF-β), produced by macrophages,

lung epithelial cells, and fibroblasts, is one of the key players [219] and indeed, higher levels of TGF-β in serum and BALF correlate with an increase in fibrosis seen on high-resolution CT scan in patients with TB six months after the start of treatment [220]. TNF-α, IL-β, and IL-17-induced neutrophil recruitment also seems to play a crucial role in the development of fibrosis [219]. MMPs appear to be involved: some MMPs reduce fibrosis, but others – perhaps counterintuitively – promote it [221]. In a Taiwanese study, patients with an MMP-1 (-1607G) gene polymorphism, leading to excessive MMP-1 production, were more likely to have moderate to advanced fibrosis on CXR one year after completion of TB treatment [222].

What are the immunological mediators and processes leading to lung damage?

Much of our recent knowledge of immunological processes in TB comes from animal models. Mice, rabbits, guinea pigs, and zebra-fish have all been used to study TB. However, none of these models completely replicate the immunopathology seen in human TB. More recently, non-human primates have also been used, exhibiting a spectrum of pathology closely resembling TB in humans [223].
For this review, we included studies done in humans, where serum and BALF markers are commonly used to assess the immunological processes in the lung. Serum measurements reflect systemic responses and do not represent what happens in individual granulomas, as was shown by a difference in gene expression patterns between granuloma and blood [201]. BALF more closely reflects responses taking place in the lung, however, even BALF only reflects processes taking place in the airways and not necessarily those in the lung parenchyma. Histology is the only way to assess the immunological processes occurring within a granuloma; however, histological samples are more difficult to obtain and, therefore, most study findings in humans are built on assumptions using available body fluid. Studies that do include histological samples cannot present longitudinal data.
When conducting our review, we searched for studies that assessed inflammatory mediators, and associated them with radiological abnormalities as a marker for pulmonary damage (Figure 4.1).

Matrix metalloproteinases

There are 23 MMPs in humans. They can be secreted by a variety of cells, including macrophages/monocytes, neutrophils and lung epithelial cells. Their generation is tightly regulated. They are not stored requiring gene transcription immediately before secretion; exceptions being MMP-8 and -9 stored in neutrophils. Once activated, they are regulated by endogenous inhibitors, called tissue inhibitors of metalloproteinases (TIMPs). Expression of MMPs is increased by prostaglandin and several cytokines (including IL-1β, IL-17 [224], TNF-α, and IFN-γ) [225]; hypoxic conditions, present in TB lesions, also increase expression and

secretion of MMP-1 through the induction of hypoxia-inducible factor 1α [226]. A recent study has demonstrated a role for platelets in MMP-1 upregulation in *Mtb*-infected monocytes, in addition to upregulation of IL-1β and IL-10 [227].

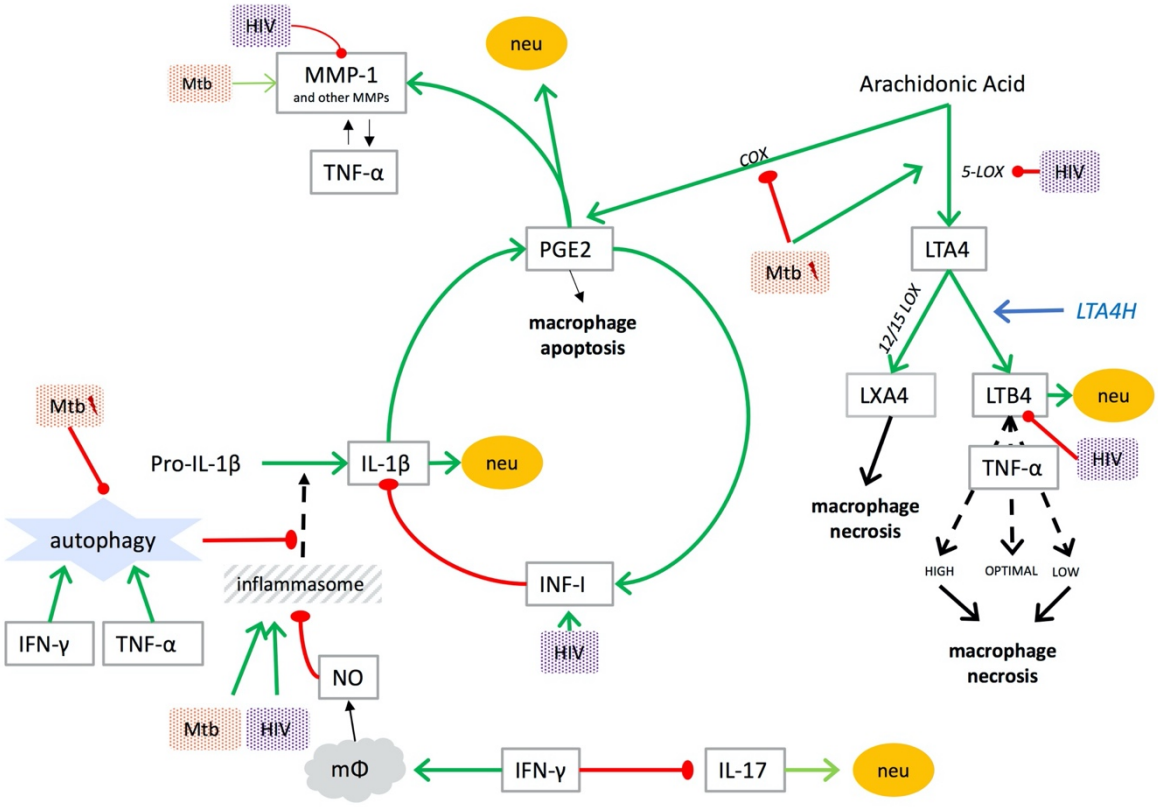


FIGURE 4.1 Mediators of lung damage in TB and interplay with HIV

↯ = virulent *Mtb*, COX = cyclooxygenase, IFN-I = type I interferon, IFN-γ = interferon gamma, IL = interleukin, LOX = lipoxygenase, LT = leukotriene, LTA4H = leukotriene A4 hydrolase, LX = lipoxin, mϕ = macrophage, MMP = matrix metalloproteinase, Mtb = mycobacterium tuberculosis, neu = neutrophil recruitment, NO = nitric oxide, PGE2 = prostaglandin E2, TNF = tumor necrosis factor. NO inhibits assembly of the NLRP3 inflammasome [228]

As described above, degradation of collagens and elastins by MMPs during active TB leads to the formation of cavities. Strong evidence of the role of MMPs in lung damage comes from studies in transgenic mice expressing human MMP-1. Wildtype mice do not express the ortholog of MMP-1 in lung and do not develop caseous necrosis or cavities in response to *Mtb*; in human MMP-1 transgenic mice, however, infection with TB leads to collagen destruction and caseous necrosis [200, 229]. MMPs also play a role in granuloma formation [230].

Several MMPs are upregulated in blood, sputum, and BALF of patients with active TB, primarily MMP-1, -3, -7, -8, and -9 [225]. MMP-1 is the dominant collagenase in TB [229]; its

secretion is driven by *Mtb* directly by activation of multiple intracellular signaling pathways and by intercellular networks [231]. Corresponding TIMPs are not similarly upregulated by *Mtb*, leading to a matrix-degrading phenotype in TB [232]. In a zebrafish model, using M. marinum to study granuloma formation, mycobacterial-derived ESAT-6 induced MMP-9 secretion, enhancing monocyte recruitment to granulomas [233, 234].
Increased levels of MMPs correlate with pulmonary damage: sputum levels of MMP-1, -2 and -8 were elevated in patients with cavities and correlated positively with the extent of infiltrates on CXR [123, 212]. Similarly, sputum levels of membrane type-1 MMP (a membrane-bound collagenase expressed on monocytes), plasma concentrations of procollagen III N-terminal propeptide (PIIINP, a degradation product of collagen type III), BALF levels of MMP-3, -7, and -8, and serum concentrations of MMP- 1, -8 and -9, correlated with more extensive CXR abnormalities in patients with TB from several different countries [235-239]. These findings suggest a central role for MMPs and extracellular matrix degradation in the development of lung damage in TB.

Neutrophils

Neutrophils are abundant in the airways of humans with active TB [240]. Their role in TB appears dichotomous: high numbers of neutrophils in the blood at the time of exposure are associated with lower likelihood of infection [241]. Conversely later in TB their numbers in blood were associated with worse patient outcomes [242, 243]. Various soluble mediators (amongst others Il-1β, IL-8, IL-17, PGE2, LTB4 and granulocyte colony-stimulating factor) promote neutrophil recruitment [244]; others, like IFN-γ and nitric oxide (NO), reduce neutrophil recruitment and survival, partly via inhibition of IL-17 [245], IL-1β and 12-lipooxygenase (12-LOX) [246].
At the time of presentation with active TB, neutrophils are associated with lung damage: a neutrophil-driven, IFN-inducible whole-blood transcript signature [247], higher blood [248, 249] and BALF [250] neutrophil counts, and higher serum levels of S100 proteins (a protein produced by neutrophils, promoting their own recruitment) [251, 252] in patients with active TB all relate with the extent of lung radiographic disease. Lung damage is thought to be contributed to by their indiscriminate killing mechanisms, which can result in significant bystander damage to surrounding host tissue. Moreover, neutrophils are the only cells that store MMPs [212], while they do not synthesize TIMPs, thus allowing for unrestrained effects of MMPs [253]. Removing infected or dying neutrophils is necessary to protect the surrounding tissue. Removal of apoptotic neutrophils by macrophages promotes subsequent killing of *Mtb*, whereas removal of necrotic neutrophils allows for mycobacterial survival and proliferation inside the macrophages. *Mtb* drives neutrophil necrosis, a process that requires neutrophil-derived reactive oxygen species (ROS) [254]. Inhibition of ROS-production could restore growth control of *Mtb* by macrophages [255].

Eicosanoids

The eicosanoids PGE2, LXA4 and LTB4 are all metabolites of arachidonic acid (AA). Cyclooxygenase (COX) converts AA into PGE2, while 5-lipooxygenase (5-LOX) generates LTA4, which is again converted into either LXA4 by 12-LOX, or LTB4 by leukotriene A4 hydrolase (LTA4H) [256]. As mentioned previously, the balance between these eicosanoids influences the mechanism of macrophage death [196]. Macrophage apoptosis leads to an early immune response with better control of the infection and minimal immunopathology, while macrophage necrosis leads to a delayed immune response, inadequate control of infection and greater immunopathology [257]. Virulent strains of *Mtb* promote LXA4 production, thereby stimulating necrosis and mycobacterial spread [196]. To our knowledge, no studies have correlated PGE2 or LXA4 with pulmonary function in human TB; one can speculate that tipping the eicosanoid-balance towards PGE2 may result in less lung damage. Findings in mice and latent TB in humans, however, show that levels of PGE2 were low early in the infection and increased later in and during active TB [258-261]. This underlines the complex and poorly elucidated role of PGE2 in TB infection and may even suggest a changing role for PGE2 during the course of the disease. LTB4, which is generated by LTA4H, has been correlated with severity of TB on CXRs in one study [262].

Cytokines

Various studies have assessed the association between cytokines (including IFN–γ and TNF–α, and several pro- and anti-inflammatory interleukins) and CXR abnormalities in TB [123, 220, 239, 247, 250, 263-274]. The different measuring methods used and the fact that several cytokines are not limited to a single effector function make comparison and interpretation challenging.

Only TNF-α and IL-1β in both blood and BALF seem to unambiguously correlate with CXR abnormalities. Higher levels of TNF-α and IL-1β correlate with the presence or size of cavities [220, 239, 263, 266] and with the extent of pulmonary involvement [123, 269]. Moreover, lower levels of these cytokines were found in patients with an early radiological response to TB treatment (improved CXR after two months of treatment) compared to those with a later (at six months) response [265]. In animal models, the effect of TNF-α seems to be dose dependent, where both high and low doses lead to tissue destruction [96, 275]. LTA4H polymorphism, and subsequently eicosanoid patterns, play a role in its regulation [96]. Both TNF-α and IL-1β affect secretion of MMPs and MMPs in their turn can play a role in the release, activation or inactivation of TNF-α and IL-1β [205]. IL-1β also associates with activation of fibroblasts [276] and the recruitment of neutrophils [244, 246], which all associate with lung damage.

Autophagy

Autophagy is an intracellular self-digestion process: cytosolic material is engulfed by a double-membrane vesicle called the autophagosome, that delivers it to lysosomes for degradation and subsequently releases the degraded products back to the cytosol.

Autophagy can be used by the host to eliminate intracellular pathogens and plays an important role in defense against *Mtb* [277]; both IFN-γ and TNF-α can induce autophagy [278]. It can also downregulate IL-1β production mediated through the inflammasome (an intracellular multiprotein complex that triggers formation of proinflammatory cytokines), by removing large inflammasome complexes or damaged mitochondria - which, through production of ROS, trigger the inflammasome [279]. Virulent *Mtb* can inhibit autophagy [280], subsequently leading to increased IL-1β production [278]. It was found that patients infected by *Mtb* strains with poor in vitro autophagy-inducing ability displayed more severe radiographic extent of disease [281]. Consequently, inducing autophagy could limit lung damage.

The modulating role of HIV

Globally, 13 percent of people with active TB who know their HIV status are co-infected with HIV-1 [172]. Although TB is also a risk factor for airflow obstruction in patients with HIV [119, 120, 282], in HIV positive patients with a low CD4 count (CD4 < 200/mm3) TB often presents with atypical CXR findings or even normal CXRs, while cavitation is fourfold less common [121]. These findings suggest that TB-related pulmonary damage might be reduced in HIV co-infected patients and the host immune response, necessary for protection against TB, is required for the development of cavities. Indeed, several of the factors previously discussed and implicated in pulmonary damage, are affected by HIV co-infection. For example, sputum levels of MMP-1, -2, -8, and -9 are reduced in HIV-TB co-infected patients, compared to patients without HIV [19, 123] as is the activity and life span of neutrophils [122]. The effect of HIV co-infection on the levels of several of the other cytokines is variable across studies and thus it is difficult to interpret a clear trend [123, 150, 283-287].

Paradoxical TB-associated immune reconstitution inflammatory syndrome (TB-IRIS) develops in approximately 18% (95% CI 16-21%) of patients on treatment for HIV-associated TB, usually within the first few weeks after starting ART [18]. It results in new or recurrent TB signs and symptoms, commonly involving the lungs, such as cough, chest pain, and worsening radiographic pulmonary infiltrates. TB-IRIS is associated with increased levels of several cytokines, particularly IL-6, TNF-α and IFN-γ [21, 59, 63, 67] and inflammasome activation [67]. It results in increased neutrophil recruitment [76], and up-regulation of MMP-1, -3, -7, -8, and -10 [19, 78, 79]. LT4AH also appears to play a role, as more severe TB-IRIS has been reported in patients with mutant (TT and CT) LTA4H genotypes [99].
These findings suggest that TB-IRIS could result in pulmonary damage and impaired lung function. To date, only one study has explored the relationship between TB-IRIS and lung function in 14 patients with HIV-associated TB, 3 of whom developed TB-IRIS [79]. The study found that an increase in MMP-8 between baseline pre-ART and 4 weeks post-ART initiation

strongly associated with impairment in lung function, but the small sample size limits definitive conclusions.

Where can we intervene to prevent or reduce lung damage?

There are several uncertain areas around therapies to prevent or limit lung damage in TB. Changes in the lungs start to develop before clinical symptoms appear [193, 288, 289], and therefore, a large proportion of lung damage may already have occurred by the time the patient presents; several mediators of lung damage may have different roles at different stages of the disease; granulomas in various stages can be present at the same time in a single individual, and only a single or a few progressive granulomas can determine the outcome of the disease. Therefore, it remains uncertain what happens for example to the contained granulomas if we systemically treat the patient with potentially immunosuppressive therapy or what the right time is to intervene (Figure 4.2).

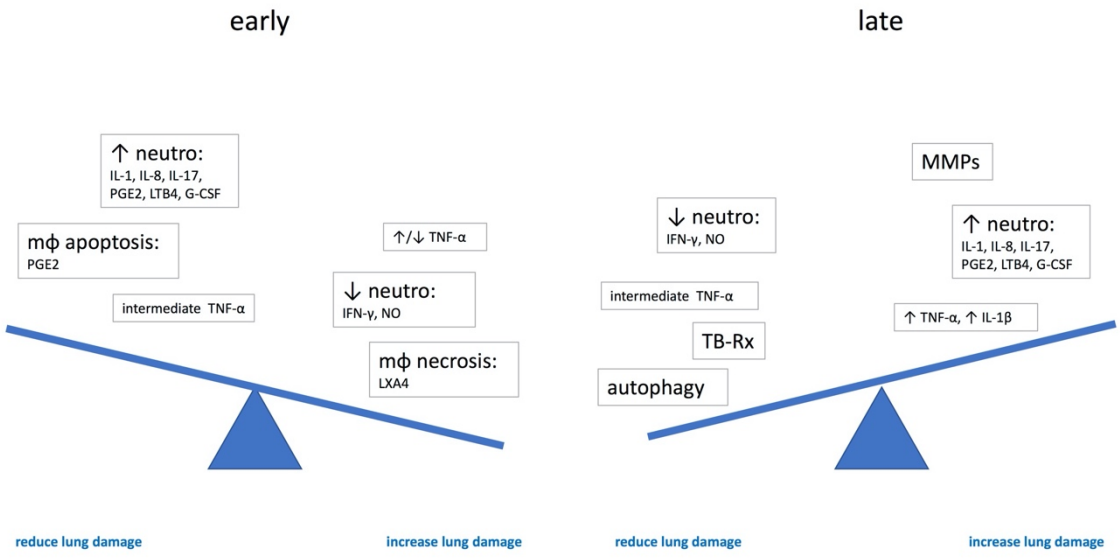


FIGURE 4.2 The potential effect of immune mediators on the development of lung damage at different stages of disease

G-CSF = granulocyte-colony stimulating factor, IL = interleukin, LTB4 = leukotriene B4, LXA4 = lipoxin A4, mϕ = macrophage, neutro = neutrophils, NO = nitric oxide, PGE2 = prostaglandin E2, TNF = tumor necrosis factor.

Antituberculous therapy as host-directed therapy

Sputum *Mtb* load is associated with systemic inflammation and, combined with pre-treatment C-reactive protein levels, inversely correlates with CXR improvement 60 days

after start of treatment [290]. Time between first TB symptoms and start of treatment [116, 173], duration of treatment [174], and smear positivity [174] are associated with impaired pulmonary function, suggesting that prompt diagnosis and treatment will limit lung damage. In addition to a direct anti-mycobacterial effect, in vitro studies suggest that some antimycobacterial agents may have immunomodulatory action. Pyrazinamide directly reduces levels of TNF-α, IL-6 and IL-1β [291], quinolones downregulate MMP-1, -3, and -9 [238], and rifampicin downregulates MMP-3 production by bronchial epithelial cells [238] and inhibits PGE2 production [292]. P-aminosalicylic acid (PAS), which is an aspirin derivate, suppresses PGE2-dependent MMP-1 production [293]. Both isoniazid (INH) and pyrazinamide (PZA) enhance autophagy [294].

Medicines used in other human diseases as host-directed therapy for TB

In an adjunctive approach to TB therapy, treatment could be supplemented with host-directed therapies. Several readily available drugs affect cytokines, MMPs or eicosanoids and therefore potentially reduce pulmonary damage (Table 4.1).

Steroids have been used as adjunctive treatment in TB for several decades [295, 296], mainly in TB meningitis, pericarditis, and TB-IRIS, even though corticosteroid use without concomitant TB treatment increases the risk of developing TB [297]. Two recent reviews concluded that there is no high quality evidence that steroid treatment significantly affects mortality or sputum conversion rate in pulmonary TB [89]. An earlier review – including mostly studies done in the 1960s and patients not on rifampicin-based TB treatment - did find a beneficial effect of steroids on radiographic resolution and regression of cavities [298]. A meta-regression analysis of 12 studies found steroids do accelerate sputum TB culture conversion [91] – which is inversely associated with development of airflow obstruction [299]; however high doses (134 mg prednisone daily) for an extended period (2 months) are required to reach clinically relevant outcomes [91]. Moreover, the only two studies in this analysis in which patients were on rifampicin-based treatment show contradicting results.

Corticosteroids inhibit various cytokines in TB (IFN-γ, TNF-α, IL-1β) and TB-IRIS (IL-6, IL-10, IL-12p40, TNF-α, IFN-γ and IP-10) [90, 139, 300, 301]. In patients with tuberculous meningitis, the effect of corticosteroids was found to be LTA4H genotype modulated, with only patients with the mutant TT genotype, leading to a higher inflammatory response, benefitting from steroid treatment [96]. In patients with TB-IRIS, however, this difference in genotype on the effect of steroid treatment was not confirmed [99]. The effect of corticosteroid treatment and TB-IRIS on pulmonary function is being assessed in a substudy of the PredART trial [43, 302].

Little evidence is available for other TNF-α blocking therapies. A trial of 16 patients with HIV-associated TB treated with etanercept (but no ART) showed a tendency to greater CXR improvement from baseline to six months compared to a placebo group, although this was not statistically significant (p=0.2) [303]. Case reports describe successful treatment of paradoxical TB reactions or TB-IRIS - involving the pleura, lymph nodes or brain - with

infliximab [42, 304, 305] or adalimumab [306, 307]. Although only one case refers to pulmonary TB-IRIS (occurring after interruption of prior anti-TNF-α treatment [306]), these case reports support the possible benefits of TNF-α blockers in the treatment of (complicated) TB. Restarting TNF-α blockers during or after TB treatment was safe and only led to one recurrence of TB in a cohort of 22 patients in Turkey followed for a median of 53 months [308].
Doxycycline is the only licensed MMP-inhibitor for use in humans. It suppresses MMP-1, -3 and -9 secretion by *Mtb* infected human macrophages and bronchial epithelial cells [123].

TABLE 4.1 Host-directed therapies potentially inhibiting lung damage and/or promoting lung repair

Host-directed therapy potentially inhibiting lung damage	**Potential mechanism**
Steroids	↓ INF-γ, TNF-α, IL-1β (and IL-6, IL-10, IL-12p40, and IP-10 in TB-IRIS) ↓ MMP-7 (in TB-IRIS)
Doxycycline	↓ MMP-1, -3, and -9
Vitamin D	↓ MMP-7 and -9 ↓ IFN-γ, IL-6, IL-10, TNF-α ↑ autophagy
Rapamycin, everolimus	↓ MMP-1 and -3 ↑ autophagy
NSAIDs	↓ PGE21 and ↑ LXA4
Zileuton	↓ 5-LOX
Phosphodiesterase-4 inhibitors	↓ TNF-α ↓ neutrophil recruitment
Metformin	↓ TNF-α ↑ autophagy
Statins	↑ autophagy
TNF-α blockers	↓ TNF-α
PGE2[1]	↑ PGE21
IFN-γ	↑ IFN-γ
Mesenchymal stromal cells	Control inflammation and mediate tissue repair

[1] The effect of inhibiting or increasing PGE2 on lung damage could vary depending on the stage of the disease

Other agents also inhibit MMPs in vitro: prednisone – in patients with TB-IRIS - suppresses MMP-7 gene expression [78], vitamin D inhibits secretion of MMP-7 and -9 [309, 310], and rapamycin (an mTOR-inhibitor and a known autophagy inducer that can also affect macrophage polarization [311]) inhibits MMP-1 and MMP-3 [312]. Use of the latter in TB is limited by the interaction with rifampicin. In mice, broad spectrum inhibition of MMPs enhances the efficacy of INH and RIF treatment [313]. Conceptually, inhibition of MMPs may lead to less pulmonary damage, but so far, no clinical trials have directly assessed this.

Currently, everolimus, a rapamycin derivate, is being tested as HDT in patients with moderate to far advanced pulmonary tuberculosis (together with vitamin D, auranofin (a gold complex with antimicrobial activity used in rheumatoid arthritis), and CC-11050 (a phosphodiesterase 4 (PDE4) inhibitor)), using rifabutin-based anti-TB treatment (ClinicalTrials.gov NCT02968927); with change in FEV1 being one of the secondary outcomes. Both rapamycin, its derivates, and vitamin D could theoretically reduce lung damage through inhibition of MMPs, although the effect of vitamin D treatment on CXR abnormalities is variable (see below). PDE4 inhibitors, in combination with INH treatment, have been shown to reduce TB-associated lung damage in rabbits [314] and pulmonary bacillary load in mice [315]. Doxycycline is being investigated for its potentially modulating effect on tissue destruction in pulmonary TB (ClinicalTrials.gov NCT02774993).

NSAIDs inhibit the enzyme cyclooxygenase (COX), thereby inhibiting PGE2 production and enhancing LXA4 production. An adjunctive role for NSAIDs in treatment of human TB has only been shown for acetylsalicylic acid in reducing PZA-induced arthralgia [316, 317] and possibly in TB meningitis [318-320]. Negative effects have been described: a Taiwanese study found an association between NSAID use (both traditional NSAIDs and selective COX-2 inhibitors) and an increased risk of active TB [321]. However, it is not clear whether this association is causative (i.e. decreased apoptosis at the very early stages of TB) or merely reflects an increased use of NSAIDs early during TB. In mice, inhibition of PGE2 by the NSAID ibuprofen was shown to affect lung pathology: inhibition early in the disease process leads to an increase in pulmonary inflammation and pathology [258], whereas inhibition later during disease decreased lung pathology and neutrophil influx [258, 322]. Increasing PGE2 by early (day one post infection) administration of exogenous PGE2 (dinoproston - normally used for induction of labor) and/or the 5-lipo-oxygenase inhibitor zileuton (used in the treatment of asthma) to IL-1 deficient mice resulted in less necrotic lung pathology by TB [259]. No studies with dinoproston or zileuton have been performed in human TB to date. A pilot study is currently investigating the effect of ibuprofen added to multi-drug resistant TB treatment on radiological improvement of TB, amongst other endpoints (ClinicalTrials.gov NCT02781909).

In in vitro models, metformin, a widely-used antidiabetic agent, has been shown to inhibit TNF production by monocytes [323], affect macrophage polarization [324], and promote autophagy [325]. It affects Th1 responses, but data are conflicting: in mice infected with TB, metformin treatment promotes the expansion of *Mtb*-specific IFN-γ secreting T cells in the lungs [325], whereas in human THP-1 cells (not infected with *Mtb*) metformin suppressed the production of Th1-related cytokines [326]. Metformin use in patients with diabetes mellitus on treatment for TB was associated with decreased mortality compared to patients using other anti-diabetic drugs in two retrospective observational cohorts [325, 327]. A retrospective cohort study of TB patients with diabetes mellitus showed that those using metformin at diagnosis and during TB treatment had fewer cavities and fewer CXR abnormalities compared to those using other anti-diabetic drugs [325]. Another

retrospective study, however, showed increased cavitatory disease in patients using metformin [327].

Vitamin D3 induces autophagy [328] and inhibits the secretion of MMP-7, -9 [309, 310], and several cytokines, for example IFN-γ, TNF-α, IL-6, and IL-10 [329, 330] in vitro. However, its effect on radiological outcomes are ambiguous: three trials comparing vitamin D3 as adjunctive therapy demonstrated no effect on CXR score [331-333] or pulmonary function [332], while one study found more CXR improvement in the vitamin-D3 treated group [334].

Statins are widely-used inhibitors of cholesterol biosynthesis. They induce autophagy in vitro [335] with broad anti-inflammatory effects, although not directly demonstrated in TB [336]. Their use has been associated with a reduced risk of developing active TB in some studies [337-339], but not in all [340]. No studies have been performed in humans assessing statins in relation to pulmonary damage in TB; in mice, statins have been found to reduce lung pathology [335]. A future study will look at the effect of pravastatin added to standard TB treatment on pulmonary function (NCT03456102).

Several studies looked at the effect of IFN-γ as adjunctive therapy for TB [341]. The studies were small, and most were performed in patients with multi-drug resistant TB. Aerosolized IFN-γ in combination with TB treatment resulted in better CXR outcomes compared to TB treatment alone. This contradicts the finding in mice, where adding IFN-γ resulted in worse pulmonary outcomes [342]. The authors conclude that IFN-γ might be beneficial as adjunctive therapy in TB, but larger trials are needed to confirm this.

Mesenchymal stromal cells are tissue-resident non-hematopoietic adult progenitor cells. They are believed to facilitate organ homeostasis and tissue repair and can modulate immune responses; they have been used in treatment of graft-versus-host-disease and autoimmune diseases [343]. In a phase 1 trial in patients with drug resistant TB, infusions of autologous mesenchymal stromal cells, 4 weeks after starting TB treatment, was safe and resulted in CXR improvement in 25/36 patients compared to 15/36 controls [344].

Conclusion

The immune mechanisms of parenchymal lung damage in human TB are complex and incompletely understood. The difference between pulmonary damage in animal models (mostly occurring as a result of primary TB) and humans (mostly occurring as a result of post-primary TB) further complicates study of this phenomenon. Processes taking place in the lung are heterogeneous, with granulomas with varying degrees of mycobacterial control existing next to each other and inflammatory cells and cytokines appearing to have different effects at different time points. MMPs seem to play an important role and consequently, inhibition of MMPs may lead to reduction in pulmonary damage, however this remains to be proven in clinical trials. Neutrophils are another key mediator of pulmonary damage, whose recruitment could potentially be inhibited by NSAIDs. The role of other effectors is less clear and better insight into their effects over the course of TB infection and disease is

needed to be able to guide potential intervention. Future studies of human TB and (host-directed) therapy should include radiographically assessed lung damage and pulmonary function as an outcome.

The effect of HIV-associated tuberculosis, tuberculosis-IRIS, and prednisone on lung function

Introduction

Tuberculosis (TB) is frequently complicated by lung function impairment. The odds of abnormal spirometric test results are 2- to 3-fold higher in patients with a history of TB compared to those without a history of TB, with both obstructive and/or restrictive impairments occurring [109, 111]. Studies of lung function in people with a history of TB found abnormal lung function in 45-87% [112, 114, 115], with even higher proportions reported in multi-drug resistant TB [116, 117]. HIV is an independent risk factor for predominantly obstructive lung function impairment [118, 120], however data on lung function impairment in HIV-associated TB are scarce, as HIV co-infected patients are frequently excluded from studies. TB-related lung damage may be less common in those co-infected with HIV, however current data are conflicting. (1) A single large study found less pulmonary impairment after TB in patients who were HIV positive patients [114], while other studies have not supported this beneficial association between HIV status and spirometric outcomes [115, 124, 345]. (2) Further, chest radiograph (CXR) findings in patients with HIV-associated TB and low CD4 counts (CD4 < 200/µl) are frequently normal or atypical [121], (3) and HIV co-infection results in lower levels of several of mediators usually implicated in inflammatory lung damage [346]. Conversely, paradoxical tuberculosis immune reconstitution inflammatory syndrome (TB-IRIS) after the start of antiretroviral therapy (ART) causes inflammation and high levels of inflammatory mediators [19, 76, 79, 163]. Two recent studies suggest TB-IRIS may cause lung function impairment [126, 127], one showing increased inflammation, as assessed by PET-CT scan, was associated with worse lung function outcomes [126]. However, in these cohorts only small numbers of patients developed pre-defined TB-IRIS, yet the authors hypothesize that increases in pulmonary inflammation can occur as part of not-clinically recognized TB-IRIS. To date, the only other study exploring the relationship between TB-IRIS and lung function found worse spirometric outcomes in the three patients who developed TB-IRIS compared to eleven controls [79].

Corticosteroids reduce inflammation and inhibit several of the immune mediators implicated in lung damage during TB [78, 90, 139, 300], and may therefore reduce lung function impairment associated with TB. Previous studies assessing this association did not find a significant effect of corticosteroid use on tests of lung function [347-350]. However,

most of these studies were performed before the introduction of rifampicin, and none included HIV co-infected patients.

In this study, we determined the prevalence of lung function impairment over time in a randomized controlled trial of patients treated for HIV-associated TB. Additionally, we assessed the effect of prednisone evaluated in comparison to placebo for prevention of TB-IRIS on pulmonary outcome in this patient group.

Methods

Design and setting

This was a substudy of the PredART trial [43], a randomized, double-blind, placebo-controlled trial assessing the efficacy of prednisone to prevent TB-IRIS. Participants were recruited from Khayelitsha, a peri-urban township in Cape Town, South Africa. They were ambulant and treated in an outpatient setting. They received either prednisone or placebo (40 mg/day for 2 weeks, followed by 20 mg/day for 2 weeks), starting within 48 hours after starting ART. Study drug could be replaced with open-label prednisone (1.5 mg/kg/day for 2 weeks, followed by 0.75 mg/kg/day for 2 weeks or longer if clinically indicated) for treatment of TB-IRIS. TB-IRIS events were adjudicated by three clinical experts using the International Network for the Study of HIV-associated IRIS (INSHI) consensus case definition [27].

Enrolment for the substudy started later than the main trial. Participants already enrolled in the main trial could enrol in the substudy from their next eligible visit, if they had not completed the main trial yet. Participants treated for multi-drug resistant TB or who prematurely discontinued TB treatment were excluded from the analysis at week 28. Successful completion of TB treatment was defined following South African National Tuberculosis Management Guidelines [103].

The substudy was approved by the same ethical committees that approved the main trial [43]. Separate written informed consent was obtained.

Procedures

Substudy visits were scheduled at week 0 (initiation of ART and study drug), 4, 12, and 28. At each visit, pulmonary symptoms (cough, dyspnoea at exertion, and dyspnoea at rest) were assessed by the trial doctor and spirometry and six-minute walk test (6MWT) were performed. Forced vital capacity (FVC) and forced expiratory volume in 1 second (FEV1) were measured using a desktop spirometer (MIR Spirolab III, Roma, Italy). Tests were performed and results interpreted using the 2005 American Thoracic Society / European Respiratory Society guidelines for spirometry, using NHANES reference ranges [351]. We defined four possible outcomes (Figure 5.1): normal lung function (FEV1/FVC $\geq$ 70% and FVC

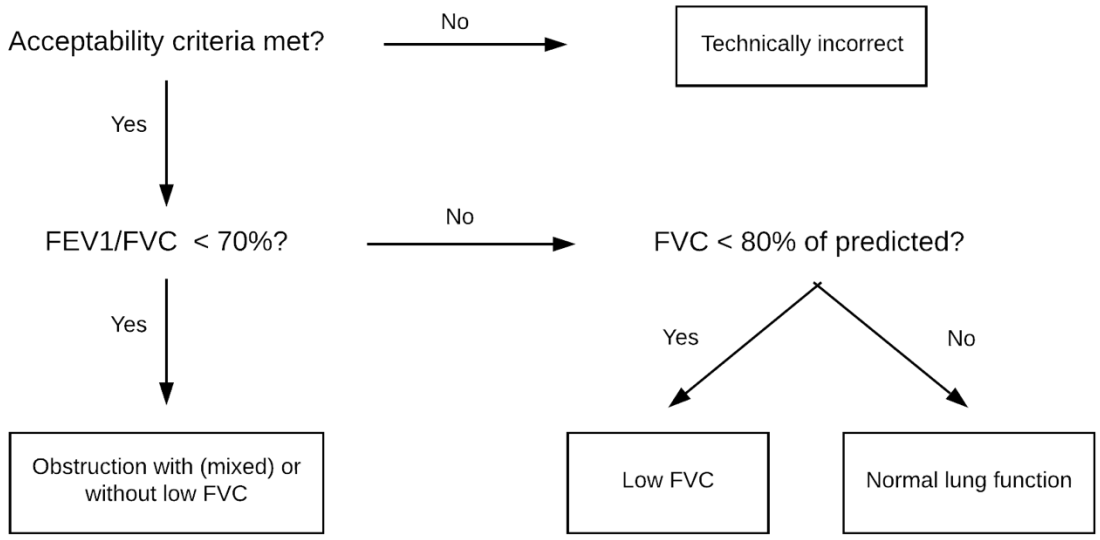


FIGURE 5.1 Definitions of different spirometry outcomes

≥ 80% of the predicted value), low FVC (FEV1/FVC ≥ 70% and FVC < 80% of the predicted value), obstructive impairment with and without low FVC (FEV1/FVC < 70% of the predicted value), and 'technically incorrect', consisting of participants who performed spirometry but did not meet criteria for interpretation (Table 5.1). 6MWTs were performed following the 2002 American Thoracic Society guidelines [352] on a 20-meter outdoor track. CXRs were performed at week 0 and 28. After completion of the study, digitized CXRs were scored by two independent blinded readers, using an adapted version of the Timika score as described by Kriel et al [353]. Where discrepancy existed, defined as a difference in score of more than 10 points, a third reader evaluated the CXR and consensus was found using a 2-1 vote.

TABLE 5.1 Acceptability criteria for the recording of FVC and FEV1 using spirometry

No artefacts	Coughing during first second of expiration
	Glottis closure
	Early termination or submaximal effort
	leak
	Obstructed mouthpiece
Good starts	Extrapolated volume < 5% of FVC or 0,15l, whichever is greater
Exhalation	Duration ≥ 6 s, plateau in the volume-time curve, or if the subject cannot or should not continue

Miller MR, Hankinson J, Brusasco V, Burgos F, Casaburi R, Coates A, Crapo R, Enright P, van der Grinten CP, Gustafsson P, Jensen R Johnson DC, Macintyre N, Mckay R, Navajas D, Pedersen OF, Pellegrino R, Viegi G, Wanger J, Force AET. Standardisation of Spirometry. E*ur Respir J* 2005; 26: 319-338

Statistical methods

Analyses were performed using Stata 14.1. For categorical variables the proportions and for continuous variables the medians with interquartile ranges (IQR) were estimated at different time points. Comparison of the Karnofsky Performance Score (KPS) between groups was done using a mixed effects proportional odds model. Correlation between CXR score and lung function was done using mixed effects regression (linear or logistic) models. Mixed effect models including a random intercept and covariates were used to model the evolution of pulmonary function over time. The effect of prophylactic prednisone on pulmonary function was tested using a test of the interaction of prednisone and visit number; participants receiving prednisone as treatment for TB-IRIS were analysed in their intention-to-treat arm (ie. study placebo or prednisone). The effect of TB-IRIS was tested using a joint test of the main effect of TB-IRIS and its interaction with visit number. A p-value of < 0.05 was considered statistically significant.

Results

Between January 2015 and February 2016, 153 participants were enrolled, 77 from the prednisone arm and 76 from the placebo arm. The flow of participants is described in Figure 5.2; baseline characteristics are summarized in Table 5.2. Seventy-one participants (46%) developed TB-IRIS, 30 in the prednisone and 41 in the placebo arm; 46 (30%) participants received open-label prednisone as treatment for TB-IRIS, 16 in the prednisone and 30 in the placebo arm.

Overall prevalence of lung function abnormalities

Symptoms

Eighty percent of participants reported cough, dyspnoea on exertion, and/or dyspnoea at rest as one of their presenting TB symptoms. At week 0, a median of 16 (IQR 15-21) days into TB treatment, 50% had one or more of these symptoms. Symptoms improved over time; however, 8% of participants who successfully completed their TB treatment at week 28 still had one or more respiratory symptom (Table 5.3).

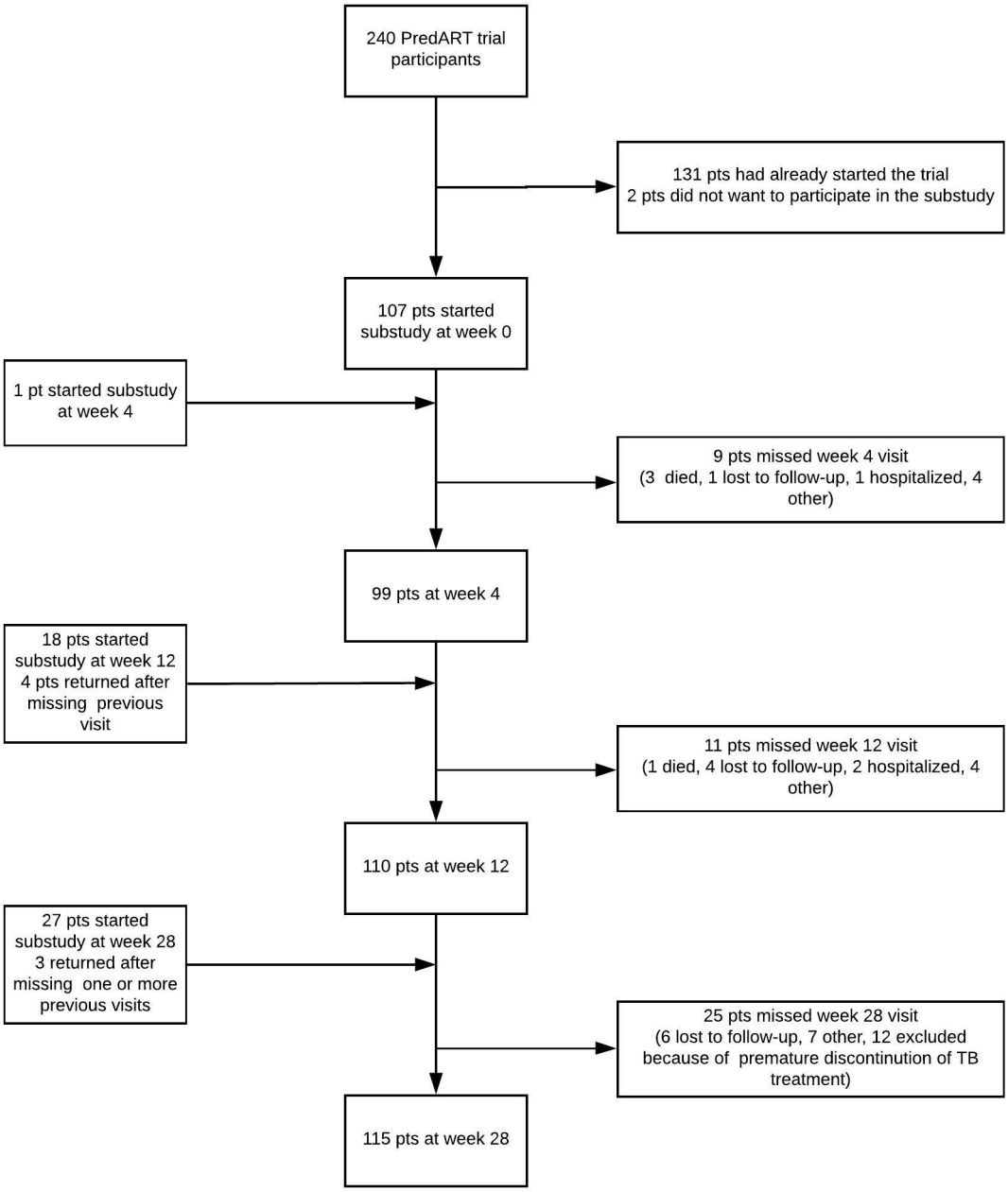


FIGURE 5.2 Number of participants per visit

Spirometry

A total of 426 spirometry tests were performed. In 15 substudy participants, spirometry was not performed at one or more time points; 5 of these participants were too ill to perform spirometry, the remaining 10 were not done for logistic reasons. Spirometic outcomes at different time points are shown in Table 5.3. The proportion of participants with a normal spirometry outcome was 26.4% at week 0, increasing to 47.4% at week 28. Low FVC was the

TABLE 5.2 Baseline characteristics and participants developing TB-IRIS and receiving prednisone as treatment according to trial arm

	Prednisone	**Placebo**
Male	49 (64%)	41 (54%)
Age (years)	38 (31-43	38 (31-44)
CD4 count (cells/µl)	46 (24-81)	50 (24-86)
HIV viral load (log_{10} copies/ml)	5.5 (5.2-5.8)	5.6 (5.3-5.9)
Extra-pulmonary TB *(n=123)*	24 (38%)	33 (55%)
Microbiologically confirmed tb	53 (70%)	59 (79%)
Previous TB	6 (8%)	8 (11%)
Time on TB treatment at week 0 (days)	16 (15-22)	16 (14-21)
Smoking status at week 0 / py *(n=127)*		
never smoked	36 (55%)	41 (67%)
ever smoked	30 (45%) / 2.2 (0.8-5.0)	20 (33%) / 3.75 (1.0-10.0)
Previous lung disease	1 (1.3%)	0 (0%)
Spirometry		
FEV1 (% of predicted)	75 (61-88)	73 (59-86)
FVC (% of predicted)	74 (66-89)	73 (65-81)
FVC/FEV1 (%)	83 (79-85)	82 (77-86)
TB-IRIS	30 (39%)	41 (54%)
Treatment with open-label prednisone for suspected TB-IRIS	16 (21%)	30 (39%)

TB = tuberculosis, pulmonary tuberculosis = participants with one or more pulmonary signs or symptoms (such as cough, shortness of breath, abnormal chest radiographs) of tuberculosis at presentation, extrapulmonary tuberculosis = participants with signs of extrapulmonary tuberculosis (such as pleural effusion or enlarged lymph nodes), microbiologically confirmed TB = participants with *Mycobacterium tuberculosis* detected on culture, with the use of the Xpert Mtb/RIF assay (cepheid), or as positive acid-fast bacilli on smear microscopy, py = packyear. data are shown as number (percentage) or median (interquartile range).

commonest abnormality. The proportion of participants with obstruction with or without low FVC was low and roughly the same over time. We found no effect of CD4 cell count recovery on change over time of spirometric outcomes (p = 0.71 for FEV1).
At week 0, 23 (21.7%) participants performed a technically incorrect test; the main reason was the inability to exhale for 6 seconds. To assess if this possibly reflected more impaired lung function, we compared six-minute walking distance (6MWD) and KPS between participants with correctly and incorrectly performed tests. On average, the participants with an incorrect test had a 6MWD which was 56 meters (95% CI 30-83; p < 0.001) shorter and a lower KPS (p < 0.001). At week 12, 13 out of 20 participants with an initial incorrect test had obstruction and/or a low FVC; with similar findings in 11 out of 16 participants at week 28.

TABLE 5.3 Symptoms, spirometric outcomes, 6MWD and CXR score in the whole study group at different time points

	week 0		week 4		week 12		week 28	
Symptoms (*N*)	*107*		*99*		*110*		*111*	
cough	40	(37.4%)	29	(29.3%)	14	(12.7%)	5	(4.5%)
dyspnoea at exertion	37	(34.6%)	27	(27.3%)	12	(10.9%)	5	(4.5%)
dyspnoea at rest	11	(10.3%)	8	(8.1%)	3	(2.7%)	1	(0.9%)
total	54	(50.5%)	43	(43.4 %)	20	(18.2%)	9	(8.1%)
Spirometry outcome (*N*)	*106*		*96*		*110*		*114*	
normal	28	(26.4%)	36	(37.5%)	50	(45.5%)	54	(47.4%)
low FVC	48	(45.3%)	42	(43.8%)	43	(39.1%)	44	(38.6%)
obstruction +/- low FVC	7	(6.6%)	7	(7.3%)	10	(9.1%)	11	(9.7%)
technically incorrect	23	(21.7%)	11	(11.5%)	7	(6.4%)	5	(4.4%)
6MWD (*N*)	*102*		*91*		*104*		*113*	
	520	(465-576)	524	(450-579)	539	(483-608)	585	(520-655)
CXR score (*N*)	*135*						*61*	
	4	(0.8-11.7)					0.9	(0-3.75)

N = total number of participants per test per visit; Symptoms total = cough, and/or dyspnea at exertion, and/or dyspnea at rest; normal = FEV1/FVC ≥ 70% *and* FVC ≥ 80% of the predicted value; low FVC = FEV1/FVC ≥ 70% *and* FVC < 80% of the predicted value; obstruction +/- low FVC = FEV1/FVC < 70%; technically incorrect = test not fulfilling criteria for interpretation; 6MWD = six-minute walk distance in meters; CXR = chest X-ray; week 0 = the start day of anti-retroviral therapy and prednisone/placebo (median 16 days after start of antituberculosis treatment). Data are shown as number (percentage) or median (interquartile range).

Six-minute walking distance

A total of 410 6MWTs were performed; 29 substudy participants did not perform a walking test at one or more time points, main reasons being painful feet or rain. Median 6MWD was 520 meters (IQR 465-576) at week 0 and 585 meters (IQR 520-655) at week 28 (Table 5.3).

Chest radiograph score

CXR scores were available for 135 participants at week 0, and for 61 participants at week 28. Possible scores ranged from 0 to 140, with higher scores indicating more CXR abnormalities. The median CXR scores were low: 4.0 (IQR 0.8-11.7) at week 0, and 0.9 (IQR 0-3.75) at week 28 (Table 2). Cavities were present in 8 (6%) participants at week 0 and 1 (2%) participant at week 28. Overall, CXR scores showed significant correlation with respiratory symptoms, 6MWD and FEV1: for an increase of 10 points in CXR score, an OR of 1.51 for symptoms (95% CI 1.13-2.03; p = 0.006) was observed (Table 5.4); the average 6MWD decreased by 21 meters (95% CI 11-31; p < 0.001); and the average FEV1 percentage of predicted decreased by 3.3 % (95% CI 1.9-4.8; p<0.001). However, in CXRs with lower scores (i.e. below 10), the

FEV1 varied markedly, and both normal and impaired lung function were seen in participants with little or no CXR abnormalities (Figure 5.3).

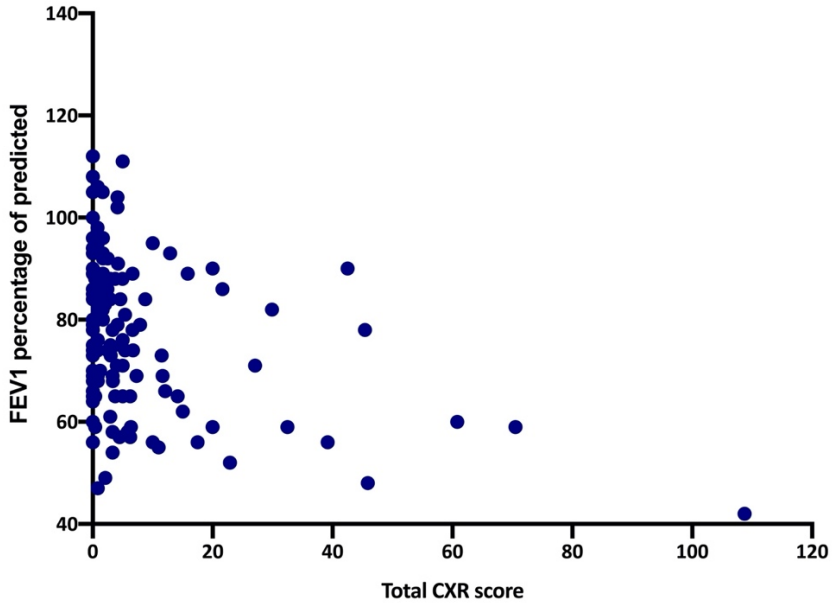


FIGURE 5.3 The association between chest X-ray score and FEV1 percentage of predicted (n = 135).

Chest X-rays were scored using an adapted version of the Timika score1): visible lung fields on the chest X-ray were divided into six zones and the percentage of affected lung in each zone and its prominent opacification type were estimated. A total score was generated by adding up the percentages of each zone and dividing the total by the number of scored zones (usually 6), adding an additional 40 points if one or more cavities > 1cm in diameter were present. Therefore, total scores could range from 0 to 140. Chest X-ray scores were statistically significantly correlated with FEV1 percentage of predicted ($p < 0.001$).

There was no significant association between CXR score at week 0 and spirometry results at week 28 (OR for normal lung function at week 28 per 10 points increase in CXR score at week 0 was 0.77 (95% CI 0.55-1.08; p = 0.13); and average FEV1 percentage of predicted at week 28 was 1.87% lower (95% CI 3.98 - -0.24; p = 0.08) for every 10-point increase of week 0 CXR score).

TABLE 5.4 Association between chest X-ray score and respiratory symptoms

	Odds ratio	p-value
Cough	1.24	0.08
Dyspnoea at exertion	1.45	0.007
Dyspnoea at rest	1.03	0.89
Total	1.51	0.006

Total = cough, and/or dyspnea at exertion, and/or dyspnea at rest. Odds ratio's are calculated for an increase of 10 units in chest-X-ray score. A p-value of < 0.05 is considered significant.

TABLE 5.5 The effect of prednisone prophylaxis on change over time of pulmonary function parameters

Change over time of six-minute walking distance (6MWD) in meters

	6MWD	95% CI	
Intercept (average 6MWD at week 0 for non-smokers)	504	484 – 523	
	Mean change in 6MWD	**95% CI**	**p-value**
Effect of smoking (ever vs never)	14	-11 – 39	0.275
	Mean change in 6MWD from week 0	**95% CI**	**p-value**
Effect of time (visit)			<0.0001
week 4	-27	-50 – -3	
week 12	32	10 – 54	
week 28	64	42 – 86	
Effect of prophylactic prednisone			0.034
week 4	42	13 – 72	
week 12	2	-26 – 30	
week 28	13	-15 – 41	

Change over time of forced expiratory volume in 1 second (FEV1) as % of predicted value

	FEV1 %	95% CI	
Intercept (average FEV1 % at week 0 for non-smokers)	76.9	73.6 – 80.2	
	Mean change in FEV1 %	**95% CI**	**p-value**
Effect of smoking (ever vs never)	-4.7	-8.8 – -0.5	0.027
	Mean change in FEV1 % from week 0	**95% CI**	**p-value**
Effect of time (visit)			<0.0001
week 4	-1.1	-4.4 – 2.1	
week 12	3.3	0.2 – 6.4	
week 28	6.8	3.6 – 10.0	
Effect of prophylactic prednisone			0.029
week 4	4.9	0.7 – 9.0	
week 12	-0.4	-4.4 – 3.5	
week 28	-1.5	-5.6 – 2.6	

Intercept and estimated coefficients with their 95% confidence intervals (95% CI) from the mixed effects regression models are listed. Data are adjusted for all other covariates presented in the table. Effect of time (visit) refers to the effect of time in the placebo arm. Because allocation to either the prednisone or the placebo arm was randomized, no adjustment for baseline variables other than smoking was done.

Effect of prednisone

There was no statistically significant difference in the change over time of symptoms (p=0.13) or CXR score (p=0.92) between the prednisone and the placebo arm. The change in 6MWD over time was statistically significantly different between the groups (p=0.03), with the largest difference at week 4: participants in the prednisone arm walked 42 (95% CI 13-72) meters further compared to participants in the placebo arm. Change over time of both FEV1 and FVC were also statistically significantly different between the two arms (p = 0.03 and p = 0.01), once again most obvious at week 4, with those in the prednisone arm having a FEV1 percentage of predicted that was 4.9% (95% CI 0.7-9.0%) higher and a FVC percentage of predicted that was 4.9% (95% CI1.3-8.5%) higher at week 4 compared to those in the placebo arm. Adjusting for the use of prednisone as treatment for TB-IRIS gave similar results (Table 5.6). Baseline lung function did not statistically significantly affect the impact of prednisone (p = 0. 56 for FEV1) (Table 5.7). At week 28, there was no longer a clear difference in either the 6MWD or FEV1 and FVC between the arms (Figure 5.4 and Tables 5.5 and 5.6).

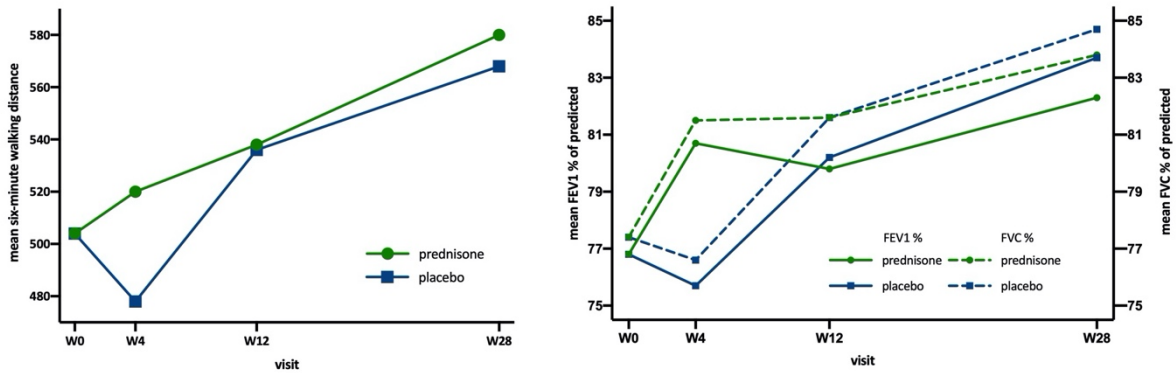


FIGURE 5.4 The effect of prednisone prophylaxis to prevent TB-IRIS on lung function.

Patients treated for HIV-associated TB received either prednisone (in green) or identical placebo (in blue) during the first 4 weeks of antiretroviral therapy. Week 0 is the day when antiretroviral therapy and prednisone or placebo were started. (A) Change over time of six-minute walk distance was statistically significantly associated with prednisone use (p = 0.034). (B) Change over time of FEV1 and FVC percentage of predicted was statistically significantly associated with prednisone use (p=0.029 & p=0.015, respectively). Graphs represent data for non-smokers. Curves for smokers are parallel.

TABLE 5.6 The effect of prednisone prophylaxis on change over time of pulmonary function parameters, adjusted for prednisone as treatment

Change over time of six-minute walking distance (6MWD) in meters

	6MWD	95% CI	p-value
Intercept (average 6MWD if all other co-variates are 0)	662	595 – 729	

	Mean change in 6MWD	95% CI	p-value
Effect of prednisone as treatment	-20	-44 - 4	0.097
Effect of smoking (ever vs never)	-45	-70 - -20	<0.001
Effect of age (per increase of one year in age at week 0)	-3	-4 – -1	<0.001
Effect of gender (female vs male)	-108	-133 – -82	<0.001
Effect of type of TB (participants without signs of extrapulmonary TB vs those with signs of extrapulmonary TB)	35	-9 – 78	0.12
Effect of HIV viral load (per log_{10} cps/ml increase at screening)	-1	-2 – -1	<0.001
Effect of CD4 count (per increase of 10 CD4 cells/µl at screening)	-1	-3 – 2	0.48
Effect of previous tuberculosis	-26	-64 – 13	0.19

	Mean change in 6MWD from week 0	95% CI	p-value
Effect of time (visit)			<0.0001
week 4	-27	-50 – -4	
week 12	34	13 – 56	
week 28	69	48 – 91	
Effect of prophylactic prednisone			0.020
week 4	44	15 – 72	
week 12	-0.03	-27 – 27	
week 28	3	-23 – 30	

Change over time of forced expiratory volume in 1 second (FEV1) as % of predicted value

	FEV1 %	95% CI	p-value
Intercept (average FEV1 % if all other co-variates are 0)	80.7	65.9 – 95.5	

	Mean change in FEV1 %	95% CI	p-value
Effect of prednisone as treatment	1.5	-3.7 – 6.8	0.57
Effect of smoking (ever vs never)	-5.6	-10.3 - -0.8	0.022
Effect of age (per increase of one year in age at week 0)	0.06	-0.2 – 0.3	0.66
Effect of gender (female vs male)	-3.5	-8.8 – 1.9	0.21
Effect of type of TB (participants without signs of extrapulmonary TB vs those with signs of extrapulmonary TB)	0.7	-8.6 – 10.1	0.88

TABLE 5.5 - continued

Effect of HIV viral load (per log_{10} cps/ml increase at screening)	0.04	-0.1 – 0.2	0.64
Effect of CD4 count (per increase of 10 CD4 cells/µl at screening)	-0.7	-1.3 – -0.2	0.008
Effect of previous tuberculosis	-14.8	-23.4 – -6.2	0.001
	Mean change in FEV1 % from week 0	**95% CI**	**p-value**
Effect of time (visit)			<0.0001
week 4	-1.5	-4.8 – 1.8	
week 12	2.9	-0.3 – 6.1	
week 28	6.4	3.1 – 9.6	
Effect of prophylactic prednisone			0.043
week 4	5.1	0.9 – 9.4	
week 12	-0.04	-4.1 – 4.0	
week 28	-1.0	-5.1 – 3.2	

Intercept and estimated coefficients with their 95% confidence intervals (95% CI) from the mixed effects regression models are listed. Data are adjusted for all other covariates presented in the table. We have not adjusted for TB-IRIS because we assume it to be on the causal pathway.

TABLE 5.6 The effect of prednisone prophylaxis on change over time of forced vital capacity (FVC) as % of predicted value

	FVC %	**95% CI**	
Intercept (average FVC % at week 0 for non-smokers)	77.3	74.4 – 80.3	
	Mean change in FVC %	**95% CI**	**p-value**
Effect of smoking (ever vs never)	-5.0	-8.7 – -1.2	0.009
	Mean change in FVC % from week 0	**95% CI**	**p-value**
Effect of time (visit)			<0.0001
week 4	-0.8	-3.7 – 2.0	
week 12	4.3	1.6 – 7.0	
week 28	7.3	4.5 – 10.1	
Effect of prophylactic prednisone			0.015
week 4	4.9	1.3 – 8.5	
week 12	-0.1	-3.5 – 3.4	
week 28	-0.8	-4.4 –2.7	

Intercept and estimated coefficients with their 95% confidence intervals (95% CI) from the mixed effects regression models are listed. Data are adjusted for all other covariates presented in the table.

TABLE 5.7 The effect of baseline spirometry outcome on the effect of prednisone on change over time of forced expiratory volume in 1 second (FEV1) as % of predicted value

	FEV1 %	95% CI	
Intercept (average FEV1 % at week 0 for non-smokers with abnormal spirometry result)	67.1	63.4 - 70.8	<0.001
	Mean change in FEV1 %	**95% CI**	**p-value**
Effect of spirometry outcome at baseline (normal vs abnormal)	25.6	20.2 - 31.0	<0.001
Effect of smoking (ever vs never)	-2.0	-6.0 – 2.0	0.33
	Mean change in FEV1 % from week 0	**95% CI**	**p-value**
Effect of time (visit)			<0.0001
week 4	-0.3	-4.5 – 3.8	
week 12	4.3	0.4 – 8.2	
week 28	9.8	5.6 – 14.0	
Effect of prophylactic prednisone			0.12
week 4	5.9	0.7 – 11.2	
week 12	0.8	-4.5 – 6.0	
week 28	-0.4	-6.1 – 5.3	
Effect of normal spirometry at baseline			0.41
week 4	-0.4	-7.6 – 6.7	
week 12	-3.0	-10.5 – 4.5	
week 28	-6.7	-15.0 – 1.6	
Effect of normal spirometry at baseline on the effect of prophylactic prednisone			0.56
week 4	-5.3	-14.2 – 3.6	
week 12	-5.7	-15.0 – 3.7	
week 28	-3.3	-13.6 – 7.1	

Intercept and estimated coefficients with their 95% confidence intervals (95% CI) from the mixed effects regression models are listed. Data are adjusted for all other covariates presented in the table. Because allocation to either the prednisone or the placebo arm was randomized, no adjustment for baseline variables other than smoking and baseline spirometry outcome was done. Only participants who had a baseline spirometry test done (n = 83) were included in this analysis.

Effect of TB-IRIS

When comparing participants who developed paradoxical TB-IRIS to those who did not, TB-IRIS was associated with a change in the presence of symptoms over time (p = 0.03), but there was no statistically significant difference in change over time of FEV1 percentage of predicted (p = 0.11), FVC percentage of predicted (p = 0.054), 6MWD (p = 0.62), or CXR score (p = 0.20) (Figure 5.5 and Tables 5.8 and 5.9).

TABLE 5.8 The effect of tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) on change over time of pulmonary function parameters

Change over time of six-minute walking distance (6MWD) in meters

	6MWD	95% CI	p-value
Intercept (average 6MWD if all other co-variates are 0)	837	711-964	

	Mean change in 6MWD	95% CI	p-value
Effect of TB-IRIS at week 0	-4	-35 - 27	0.79
Effect of smoking (ever vs never)	-44	-69 – -19	0.001
Effect of age (per increase of one year in age at week 0)	-3	-4 – -2	<0.001
Effect of gender (female vs male)	-112	-138 – -86	<0.001
Effect of type of TB (participants without signs of extrapulmonary TB vs those with signs of extrapulmonary TB)	30	-14 – 74	0.19
Effect of HIV viral load (per $\log_{10}$ cps/ml increase at screening)	-32	-50 - -13	0.001
Effect of CD4 count (per increase of 10 CD4 cells/µl at screening)	-2	-4 – 1	0.21
Effect of previous tuberculosis	-21	-60 – 18	0.28

	Mean change in 6MWD from week 0	95% CI	p-value
Effect of time (visit)			<0.0001
week 4	-16	-45 – 14	
week 12	40	13 – 67	
week 28	72	44 – 100	
Effect of TB-IRIS			0.68
week 4	-21	-54 – 13	
week 12	-11	-44 – 21	
week 28	-7	-40 – 27	
Effect of prophylactic prednisone			0.036
week 4	41	13 – 70	
week 12	0	-27 – 26	
week 28	4	-22 – 31	

TABLE 5.8 - continued

Change over time of forced expiratory volume in 1 second (FEV1) as % of predicted value

	FEV1 %	95% CI	p-value
Intercept (average FEV1 % if all other co-variates are 0)	83.9	57.5-110.3	

	Mean change in FEV1 %	95% CI	p-value
Effect of TB-IRIS at week 0	3.2	-2.7 – 9.2	0.29
Effect of smoking (ever vs never)	-5.9	-10.6 – -1.1	0.02
Effect of age (per increase of one year in age at week 0)	0.04	-0.2 – 0.3	0.76
Effect of gender (female vs male)	-3.6	-9.0 – 1.8	0.19
Effect of type of TB (participants without signs of extrapulmonary TB vs those with signs of extrapulmonary TB)	0.7	-8.6 – 10.1	0.88
Effect of HIV viral load (per $\log_{10}$ cps/ml increase at screening)	-0.5	-4.5 – 3.4	0.79
Effect of CD4 count (per increase of 10 CD4 cells/µl at screening)	-0.8	-1.3 – -0.2	0.005
Effect of previous tuberculosis	-14.8	-23.4 – -6.1	0.001

	Mean change in FEV1 % from week 0	95% CI	p-value
Effect of time (visit)			<0.001
week 4	1.4	-3.0 – 5.7	
week 12	6.3	2.3 – 10.4	
week 28	8.1	3.9 – 12.3	
Effect of TB-IRIS			0.06
week 4	-4.7	-9.4 – -0.1	
week 12	-6.0	-10.5 – -1.4	
week 28	-2.8	-7.6 – 2.0	
Effect of prophylactic prednisone			0.07
week 4	4.4	0.2 – 8.7	
week 12	-0.1	-4.8– 3.2	
week 28	-1.3	-5.5 – 2.8	

Intercept and estimated coefficients with their 95% confidence intervals (95% CI) from the mixed effects regression models are listed. Data are adjusted for all other covariates presented in the table.

Sixteen participants developed TB-IRIS without any respiratory signs or symptoms. Exclusion of these non-pulmonary IRIS cases from the analysis did not affect the results (data not shown).

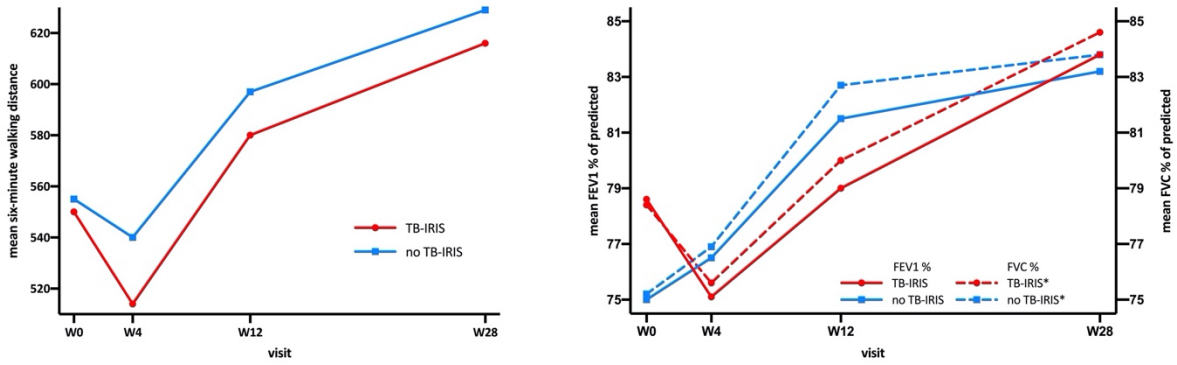


FIGURE 5.5 The effect of the development of tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) on lung function.

Patients with TB-IRIS (red) are compared to those without TB-IRIS (blue). (A) Change over time of six-minute walk distance was not statistically significantly associated with TB-IRIS (p = 0.62). (B) Change over time of FEV1 and FVC percentage of predicted was not statistically significantly associated with TB-IRIS (p = 0.11 & p = 0.054, respectively)). Graphs represent data for male non-smokers in the placebo arm of age 40 who have pulmonary TB, an HIV viral load at screening of 800000 copies/ml, a CD4 at screening of 100 cells/µl and did not have previous TB. The difference between TB-IRIS and no TB-IRIS were similar for other patient profiles.

Discussion

We assessed pulmonary function in a cohort of patients with HIV-associated TB at high risk for TB-IRIS, enrolled in a trial investigating the efficacy of prophylactic prednisone in preventing TB-IRIS.

Respiratory symptoms were common early during TB treatment and abnormal spirometry (low FVC and airflow obstruction with and without low FVC) was found in 66% of participants with acceptable spirometry. At the end of TB treatment, symptoms persisted in 8% and abnormal spirometry in 50% of participants. The proportion of abnormal spirometry results is higher than expected for either the general or HIV-infected population [109, 119], but is comparable to results from other studies in HIV-associated TB patients [114, 115, 127].

In our trial, open-label treatment of TB-IRIS with prednisone was allowed. This resulted in 30/76 participants in the placebo arm receiving prednisone for the treatment of TB-IRIS and the majority of the participants who developed TB-IRIS receiving prednisone, either as prophylaxis, as treatment, or both, with prednisone treatment given to the more severe cases of TB-IRIS. This limits our evaluation of the individual effects of both prednisone as well as TB-IRIS on lung function.

TABLE 5.9 The effect of tuberculosis-associated immune reconstitution inflammatory syndrome (TB-IRIS) on change over time of forced vital capacity (FVC) as % of predicted value

	FVC %	95% CI	p-value
Intercept (average FVC % if all other co-variates are 0)	80.3	55.7 – 104.8	<0.001
	Mean change in FVC %	**95% CI**	**p-value**
Effect of TB-IRIS at week 0	2.9	-2.6 – 8.3	0.30
Effect of smoking (ever vs never)	-5.9	-10.1 – -1.6	0.007
Effect of age (per increase of one year in age at week 0)	0.1	-0.2 – 0.4	0.45
Effect of gender (female vs male)	-2.7	-7.8 – 2.3	0.29
Effect of type of TB (participants without signs of extrapulmonary TB vs those with signs of extrapulmonary TB)	3.7	-5.0 – 12.5	0.40
Effect of HIV viral load (per $\log_{10}$ cps/ml increase at screening)	-1.0	-4.6 – 2.7	0.61
Effect of CD4 count (per increase of 10 CD4 cells/µl at screening)	-0.7	-1.2 – -0.2	0.009
Effect of previous tuberculosis	-10.6	-18.7 – -2.6	0.010
	Mean change in FVC % from week 0	**95% CI**	**p-value**
Effect of time (visit)			<0.0001
week 4	1.7	-2.0 – 5.5	
week 12	7.5	3.9 – 11.0	
week 28	8.7	5.0 – 12.3	
Effect of TB-IRIS			0.026
week 4	-4.4	-8.5 – -0.4	
week 12	-5.7	-9.7 – -1.8	
week 28	-2.5	-6.7 – 1.7	
Effect of prophylactic prednisone			0.040
week 4	4.5	0.7 – 8.2	
week 12	-0.4	-4.0 – 3.1	
week 28	-0.8	-4.4 – 2.9	

Intercept and estimated coefficients with their 95% confidence intervals (95% CI) from the mixed effects regression models are listed. Data are adjusted for all other covariates presented in the table

Within these limitations, we did not find an effect of TB-IRIS on spirometric lung function over time. This contradicts findings of two recent studies [126, 127] that hypothesized that TB-IRIS-like increases in inflammation may lead to decreased lung function, which may occur in patients with HIV-associated TB initiating ART, even in the absence of clinically overt TB-IRIS. It is possible that in the present study, mild TB-IRIS did not result in sufficient additional pulmonary inflammation to affect long-term respiratory outcomes, whereas in severe TB-IRIS the effect was ameliorated by treatment with prednisone.

We found prophylactic prednisone affected change over time of both 6MWD and FEV1 and FVC, primarily at week 4, when participants completed their study prednisone, potentially by preventing TB-IRIS. Consequently, the higher proportion of participants with TB-IRIS in the placebo arm may be responsible for the demonstrated favourable effect of prophylactic prednisone on lung function. Additionally, prednisone can directly improve exercise performance [354] and FEV1 in other disease processes, for example acute exacerbations of chronic obstructive pulmonary disease (COPD) [355]. Ravimohan et al [126] found that decreased lung function after 4 weeks of TB treatment, impacts negatively on long-term lung function, and Auld et al [127] found similar associations, but only in severe lung function declines. In the current study, despite an increase in lung function at week 4, we did not observe any long-term effects of prophylactic prednisone on lung function. This could be due to type 2 error, with large proportion of participants in the placebo arm receiving prednisone as treatment for TB-IRIS. Alternatively, prednisone may have been given too late in the disease process and prescribed only after lung damage had already occurred.

We found a high proportion of participants with technically incorrect spirometry results early on during treatment – a finding not reported previously. In studies performed in participants without TB, in the later stages of TB treatment or after TB treatment, 2-30% did not perform spirometry correctly and these patients were subsequently excluded [109, 112, 114, 115, 120, 126, 356]. We considered that the participants who did not perform spirometry correctly might include those with worse pulmonary status: for example, in severely ill patients, and in those with significant cough and/or shortness of breath spirometry is technically challenging. This hypothesis was supported in our study by finding that the majority of technically incorrect spirometric results were due to inability of patients to exhale for six seconds. Further, the association with a shorter 6MWD and a lower KPS, and abnormal lung function in the majority of these participants at follow-up spirometry testing adds weight to this argument. Thus, excluding these participants from the analyses may underestimate the burden of lung function impairment in TB. In our study, when technically incorrect were included as abnormal, the percentage abnormal tests at baseline increased from 66% to 74%.

In keeping with published data on HIV-associated TB patients with a low-CD4 count [121], only a small proportion of participants demonstrated extensive CXR abnormalities, while cavitation was uncommon. Our finding of a negative association between CXR score and FEV1, although statistically significant, needs to be interpreted with caution. Although a high score is unsurprisingly associated with lower FEV1, a low score does not appear to rule out significant abnormality in FEV1. Several other studies, using many different scoring methods, have reported the relationship between FEV1 and CXR score in TB [112, 115, 124, 173, 177]. However, all studies describe more severe CXR abnormalities than our present study, and few included HIV positive patients. Those that did, chose not to report data on

HIV patients specifically, or claimed the numbers were too small for meaningful sub-analysis. Our observation that the presence of a normal CXR in HIV-associated TB patients with a CD4 count < 100 cells/µl does not exclude lung function abnormalities, is likely explained by the insensitivity of CXR to detect changes responsible for the reduced FEV1, for example small airways and subtle parenchymal abnormalities.

Besides the considerable overlap between participants developing TB-IRIS and participants prescribed prednisone to treat TB-IRIS, our study has other limitations. First, as consequence of the substudy commencing after the main trial, we do not have complete data on all participants. Second, we do not have reliable information about the time between the start of symptoms and the start of TB treatment, with longer duration of symptoms being a risk factor for pulmonary impairment [115, 357]. Third, normal values for 6MWD in our population are lacking. Most participants walked relatively far, possibly because of the relatively short duration of illness and possibly younger age of participants when compared with other chronic lung diseases. Finally, the use of the NHANES reference range (derived in North American populations) may have resulted in an overestimate of lung function impairment, as normal values for FEV1 and FVC tend to be higher than those for African populations [358].

In conclusion, we found that lung function impairment is common in patients with HIV-associated TB. Prednisone to prevent TB-IRIS improved lung function at week 4, possibly by reducing TB-IRIS, however, the 28-day course of prednisone did not improve lung function from week 12 onwards in patients with CD4 counts < 100 cells /µl. Overlap between the groups through the development of TB-IRIS and subsequent use of prednisone as treatment, limits our ability to make definitive conclusions. Prednisone remains recommended to prevent TB-IRIS in this population based on the findings of the main PredART trial [43], despite this study being unable to demonstrate long term benefits in lung function. Further studies, using PET-CT imaging and other biomarkers of inflammation and lung damage in TB [346] are needed to better understand the pathogenesis of lung function impairment in HIV-associated TB.

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Conclusion

In the PredART trial [43] we enrolled 240 patients with HIV-associated tuberculosis at high risk of TB-IRIS (time between initiation of antituberculosis treatment and ART < 30 days; CD4 count ≤100 cells/µl) and randomly assigned them to receive either prednisone or placebo during the first four weeks of ART. This trial showed that prophylactic prednisone reduced the incidence of paradoxical TB-IRIS, without an excess of adverse events. Moreover, it provided us with the unique opportunity to further assess certain aspects of HIV-associated TB, TB-IRIS and prednisone. During the trial, we collected extensive clinical information from each participant with regards to the presentation of TB-IRIS; whole blood, plasma and peripheral blood mononuclear cells were isolated and stored at -80 degrees for every participant enrolled in the trial at week 0, 2, 4, and 12 with additional samples taken and stored at the time of suspected TB-IRIS; and I developed a protocol to add lung function testing as a substudy to the trial. With these data I was able to systematically evaluate the clinical presentation and diagnosis of TB-IRIS; assess aspects of the immunopathology of TB-IRIS as well as the mechanism by which prednisone reduces the incidence of TB-IRIS; and assess the effect of TB-IRIS and prednisone on lung function in patients with HIV-associated tuberculosis. These topics formed the basis of this thesis.

Currently, the diagnosis of TB-IRIS relies on characteristic clinical features synthesized as the INSHI case definition [27]. There is no confirmatory laboratory test. In chapter two I applied latent class analysis to model a gold standard for TB-IRIS. I used available data of 217 participants from the PredART trial – of whom 41% developed TB-IRIS - and combined them into several latent class models. I initially included baseline participant characteristics (sex, age, details about current and previous TB episodes); INSHI major criteria; all individual symptoms of the INSHI minor criteria; any additionally documented signs and symptoms provided they occurred in more than 10 (4%) participants and could feasibly be related to TB-IRIS; baseline (immediately prior to starting ART) and follow-up laboratory variables (hemoglobin, leucocyte count, creatinine clearance, alanine transferase, alkaline phosphatase, C-reactive protein (CRP), CD4 count and HIV viral load); signs and symptoms that were identified as possibly related to TB-IRIS during the TB-IRIS adjudication process; and baseline urine lipoarabinomannan (LAM). The final model included the following parameters: respiratory symptoms, night sweats, INSHI major criteria 1, 2, and 4, maximum CRP > 90 mg/l, maximum heart rate > 120/min, maximum temperature > 37.7 ^{0}C, and pre-ART CD4 count < 50 cells/µl. The model estimated a TB-IRIS incidence of 43% and had optimal goodness of fit (X2 = 337, p = 1.0). With the model-derived gold standard for TB-IRIS, I could estimate sensitivity and specificity of individual signs and symptoms for TB-IRIS and compute a model-predicted probability of TB-IRIS for each participant. Using the latter, I first assessed the performance of the INSHI case definition and found it identifies TB-IRIS with a reasonable accuracy: a sensitivity of 0.77 and a specificity of 0.86. Next, I constructed

several adapted case definitions, aiming to either simplify the case definition or replace the sometimes subjective INSHI minor criteria with objectives measures like CRP elevation, fever, and/or tachycardia, thereby creating a definition that is more robust and standardised, which is advantageous if it is to be used as an endpoint in clinical trials. I compared the diagnostic accuracy of the adapted case definitions with that of the INSHI case definition. An amended definition in which I replaced all the minor INSHI criteria with the objective variables CRP elevation, fever, and/or tachycardia resulted in a definition with improved sensitivity (0.89) without a loss of specificity (0.88).
Last, I assessed the utility of CRP as a rule-out test for TB-IRIS. A normal CRP (< 10 mg/l) can unsurprisingly be used to rule out TB-IRIS. Higher cut-off values also have value in ruling out TB-IRIS.

TB-IRIS associates with hypercytokinemia [59], but the immunopathological mechanisms underlying it are not completely understood. Initial studies focused on restoration of mycobacteria specific T cell responses as an underlying mechanism. Recently, attention has shifted towards the innate immune system [60, 64]. In chapter three, I elucidated some of the mechanisms underlying TB-IRIS. I measured a panel of 31 chemo- and cytokines in samples from PredART participants taken at week 0 and week 2 and compared concentrations of chemo- and cytokines at both time points between participants who did and did not develop TB-IRIS. I found no difference in cytokine profile at baseline between the two groups. I confirmed that TB-IRIS is associated with higher concentrations of several chemo- and cytokines at week 2. These chemo- and cytokines were related to both the innate and the adaptive immune system.

I next assessed how prednisone reduces the risk of TB-IRIS. It is unlikely that prednisone only suppressed TB-IRIS symptoms in our trial: the median time to TB-IRIS in those who developed TB-IRIS was similar in both the prednisone and the placebo group. Moreover, prednisone also reduced the incidence of more severe TB-IRIS, judged from the number of patients with TB-IRIS fulfilling at least one INSHI major criterion or receiving prednisone treatment for TB-IRIS. Also, there did not appear to be “breakthrough cases” when the 28-day course of prednisone was stopped suggesting that the course of prednisone had prevented the onset of the syndrome, rather than merely suppressing symptoms. In an earlier TB-IRIS treatment trial prednisone – when compared to placebo - was shown to reduce the concentrations of several chemo- and cytokines over 4 weeks of treatment [139]. To assess the possible mechanism by which prophylactic prednisone reduces TB-IRIS, I compared chemo- and cytokines at week 2 between participants in the prednisone and in the placebo arm. I showed that prednisone was associated with a reduction in plasma concentrations of the chemo- and cytokines found to be increased in TB-IRIS.

LTA4H is an enzyme that hydrolyses LTA4 into the pro-inflammatory LTB4. It is regulated by a single nucleotide polymorphism (SNP) close to the promotor region of the *lta4h* gene. The

wildtype genotype (CC) is associated with lower concentrations of LTA4H, whereas the double mutant genotype (TT) is associated with increased LTA4H activity [98]. LT4B is a strong attractor of neutrophils and macrophages to sites of inflammation. Therefore, one could expect *lta4h* genotype to both associate with inflammation in TB-IRIS and influence the plasma chemo- and cytokine profile. I compared *lta4h* genotype between participants with and without TB-IRIS and assessed the association between *lta4h* genotype and concentrations of chemo- and cytokines. I did not find an association between genotype and the development of TB-IRIS, nor did I find an association between genotype and cytokine profile at either week 0 or week 2. A recent study suggests that the efficacy of corticosteroids as adjunctive treatment in tuberculous meningitis might depend on *lta4h* genotype [96]. In the last part of chapter three I evaluated if this correlation was also true for our trial cohort but could not find a statistically significant association between the efficacy of prednisone in preventing TB-IRIS and genotype. Possible reasons for not finding an association with *lta4h* genotype and either TB-IRIS or the efficacy of prednisone include the low frequency of this allele in our study cohort; the difference between pulmonary tuberculosis and tuberculous meningitis; or the effect of HIV co-infection.

Residual pulmonary impairment is common after treatment for tuberculosis [112, 114, 115]. Lung function data in patients with HIV-associated tuberculosis are scarce, especially in the context of TB-IRIS and prophylactic prednisone. In chapter five, I determined the prevalence of lung function abnormalities in patients with HIV-associated tuberculosis and CD4 counts ≤ 100 cells/µl and assessed the effect of prophylactic prednisone and the development of paradoxical TB-IRIS on pulmonary function. I performed spirometry, six-minute walk test, and chest radiography at week 0, 4, 12, and 28 in 153 participants of the PredART trial. I have not assessed reversibility of obstruction following bronchodilators nor measurements of gas exchange like diffusion capacity of the lungs for carbon monoxide (DLCO). I found abnormal spirometry measurements in 67% of participants at week 0 and 53% at week 28; low forced vital capacity was the commonest abnormality. A high proportion of participants (22%) had a technically incorrect spirometry test at baseline; I considered that among these participants were those with worse pulmonary status. Excluding them from the analyses could underestimate the burden of lung function impairment in TB. In our study, including technically incorrect tests as abnormal increased the percentage abnormal tests at baseline from 67% to 74%.

Chest radiographs showed little or no abnormalities in the majority of participants. However, I found that the presence of a normal chest radiograph does not exclude lung function abnormalities in patients with HIV-associated TB and a CD4 count < 100 cells/µl. Since inflammation plays an important role in lung damage in tuberculosis, I hypothesized that prednisone may reduce lung function impairment, while TB-IRIS could result in more lung function impairment. However, I found neither prophylactic prednisone as used in our study nor the development of TB-IRIS significantly affected week 28 pulmonary outcome. I

did find that participants in the prednisone arm had a 42 meters greater six-minute walk distance and a 4.9 % higher percentage of predicted forced expiratory volume in 1 second at week 4; these differences were no longer significantly different from week 12 onwards. Whether a longer duration of corticosteroid treatment does affect post-tuberculosis lung damage could be assessed in a future clinical trial. Contrasting our hypothesis and findings of two recent studies [126, 127] that hypothesized that TB-IRIS-like increases in inflammation may lead to decreased lung function, TB-IRIS did not significantly impair lung function outcome at any time point in our study cohort.

The main strength of our trial cohort for all these substudies was its uniformity, with similar baseline characteristics both between the prednisone and the placebo arm owing to randomisation, but also largely between the TB-IRIS and the no TB-IRIS group. A similar median time to TB-IRIS facilitated the analyses of the immunopathology of TB-IRIS. The main limitation of the cohort for our substudies was the overlap or association between the use of prednisone and the development of TB-IRIS, making it challenging to analyze the independent effect of prednisone or TB-IRIS on either immune markers or lung function outcomes separately. Moreover, in the analysis of the immune markers, the use of data dimensionality reduction techniques like principal component analysis (PCA) may have helped in visualizing which immune markers cluster together, allowing for better insights as to how individual immune mediators are coordinated in their response during TB-IRIS. Lastly, the lung function substudy only started recruitment after the main trial had already started. Power calculations - based on an expected mean % FEV1 of 70% of predicted and a SD of 17% at 6 months and an expected effect of prednisone of 12% - showed an expected power of >90% with the inclusion of 90 patients in each arm. However, because of the reason mentioned above, we were unable to enrol the calculated number of participants.

The diverse topics covered in this thesis illustrate the many aspects associated with TB-IRIS. By covering the different aspects of TB-IRIS this thesis aimed to provide a more complete understanding of its pathophysiology. Comparing outcomes between different HIV-associated TB study cohorts will be necessary but is often still hampered by the various definitions used to identify patients with TB-IRIS. Uniformity between studies could improve when all studies use the same definition for TB-IRIS, preferably one that is less dependent on (often subjective) patient symptom reporting. Therefore, I recommend using our amended case definition next to the existing INSHI definition to define TB-IRIS when conducting studies with TB-IRIS as an endpoint. A definition including only INSHI major criteria had the highest specificity. This definition could be used in studies where one only wants to identify participants with a very high likelihood of TB-IRIS.

One of the aspects referred to above - which has long been underappreciated in tuberculosis and TB-IRIS, but recently gained more attention – is post-tuberculosis lung damage. TB can leave the lungs permanently scarred, turning it from a treatable

communicable disease into a chronic disease. With abnormal lung function present in 45-87% of patients treated for TB [112, 114, 115] and roughly 10 million new TB cases occurring each year [11], post-tuberculosis lung disease may be one of the most important causes of chronic lung disease globally. There is a need for studies investigating ways to reduce lung function impairment in patients with tuberculosis, both in patients with and without HIV co-infection. Host immune responses play a key role in the development of pulmonary damage in TB; important mediators involved are matrix metalloproteinases (MMPs), the balance of eicosanoids in releasing pro- and anti-inflammatory cytokines, and the fate of neutrophils and macrophages (apoptosis vs necrosis) – as reviewed in chapter four. Combined immunology and lung function studies could shed more light on the timing and mechanisms of lung function damage in tuberculosis and the possibility of timely intervention to prevent lung function impairment. Studies doing so are rare, especially in patients co-infected with HIV. A small study of 14 patients with HIV-associated TB showed that increasing concentrations of MMP-8 after initiation of ART were associated with a lower FEV1 after TB treatment completion [79]. A recent study assessed the role of genes associated with the inflammasome in inflammation and lung function in 102 patients with HIV-associated TB [359]. The authors found that patients carrying the minor allele of the *NLRC4* gene had lower pre-ART concentrations of IL-18, IL-6 and TNFα as well as better lung function (measured as the percentages of predicted of FEV1 and FVC) during treatment up to 48 weeks after starting ART. Moreover, they found higher plasma IL-6 concentrations and higher expression of IL-1β, IL-18, IL-6 and IFN-γ mRNA were associated with worse lung function at baseline and/or 4 weeks after ART initiation. A large, multi-country cohort study, prospectively assessing lung function in 1500 patients with newly diagnosed TB up to 2 years after completion of TB treatment, is currently ongoing [360]. As part of the study, multiple blood, sputum and urine samples will be taken and stored with the aim to identify pathogenic mechanisms underlying poor TB treatment outcomes.

Knowing which factors contribute to lung damage in TB can guide interventions to prevent its development. Host-directed therapies (HDT) are increasingly being investigated for their role in preventing lung damage in TB. A wide range of drugs, mostly designed for other indications, has been assessed, including TNF-α blockers, doxycycline, NSAIDs, phosphodiesterase inhibitors, metformin and IFN-γ (reviewed in chapter four). Trials investigating the effect of azithromycin, everolimus, auranofin (an oral gold salt), vitamin D3, statins, and NSAIDs on lung function are currently ongoing (ClinicalTrials.gov). Corticosteroids are the oldest and most investigated of these drugs. They reduce inflammation and inhibit various cytokines in TB, like IL-1β , IFN-γ and TNF-α, [90, 300, 301] and may therefore reduce post-tuberculosis lung damage. Several studies have assessed the effect of corticosteroids on lung function in TB [347-350]. These studies did not find a significant effect of corticosteroid use on lung function. However, most of them were done in the 1960s, before the introduction of rifampicin, and none of them included HIV co-infected patients. I assessed the effect of prednisone on various cytokines as well as on lung

function in our trial cohort. In this selected group of patients with HIV-associated TB and a high incidence of TB-IRIS, I found prednisone use (given to prevent TB-IRIS) was associated with lower plasma concentrations of IL-1ra (possibly mirroring IL-1β), IL-6 and CXCL-10; prednisone at the dose used in this trial did not result in better long term lung function outcomes. Data of these two substudies could be used in future analyses to assess possible associations between chemo- and cytokines and CRP, CD4 count and HIV viral load, lung function and prednisone use. Because of the association between prednisone use and TB-IRIS and the effect of TB-IRIS on both chemo- and cytokines and possibly lung function, the independent effects on the outcomes will however be difficult to dissect. Moreover, in order to better understand the role of specific cytokines and chemokines in TB-IRIS, future studies could consider including antagonists of pro-inflammatory cytokines in their analysis. Simultaneously assessing changes in the frequency and immunophenotype of relevant circulating immune cells like monocytes or T-cells, rather than only analyzing plasma analytes, can also contribute to a better understanding of the cellular origin of the various chemo- and cytokines.

Regardless, I think lung function tests should be added as an endpoint in all clinical trials assessing interventions - including HDTs - for tuberculosis and TB-IRIS. Through this we could get a full understanding of the short- and long-term effects of the intervention; microbiological endpoints – as is often the case for TB studies – or the occurrence of TB-IRIS alone should no longer be the sole endpoint of a study. Spirometry is relatively cheap and easy to perform. However, it should be kept in mind that performing spirometry early during treatment for tuberculosis can be challenging. Patients are often severely ill, with significant cough and/or shortness of breath. Future studies should look at the possibility to amend quality criteria in this group of patients to not exclude those who are unable to perform spirometry properly because of acute illness.
Advanced HIV infection is correlated with a reduced DLCO [282], even in patients with normal spirometry values [361]. In a group of patients with COPD, patients with previous TB had significantly decreased DLCO compared to those without a history of TB [362]. The same is true for patients with bronchiectasis and previous TB [363]. Therefore, assessing gas exchange measurements – although not always easily accessible - next to spirometry may add valuable information.

Genetic studies are another aspect to prioritize in TB research. Various genes have been associated with the risk of tuberculosis [364] or susceptibility or rate of progression in HIV [365]. Genetic factors associated with TB-IRIS are still poorly elucidated. Two small studies investigated single nucleotide polymorphisms (SNP) in genes encoding cytokines, chemokines and their receptors. One study from Australia found polymorphisms in the IL-6 and TNF-α genes associated with risk for mycobacterial IRIS [366]. Another study, including patients from Cambodia and India, found TB-IRIS risk associated with polymorphisms in TNF-α, IL-18, vitamin D receptor, and natural resistance-associated macrophage protein 1 genes

[367]. A recent study including 88 patients with HIV-associated TB from Brazil showed increased risk of TB-IRIS in patients with polymorphisms in a gene for NK cell receptors and certain human leukocyte antigen (HLA) haplotypes [368]. LTA4H polymorphism has been associated with TB-IRIS severity in an Indian cohort, with more severe IRIS happening in patients with mutant genotypes, although the overall incidence of TB-IRIS was not genotype dependent [99]. In tuberculous meningitis (TBM) *lta4h* genotype has been associate with both outcome of the disease and efficacy of corticosteroids in its treatment [96]. This led to a trial, currently ongoing, to further assess the effect of dexamethasone in TBM: in HIV-uninfected patients, *lta4h* genotype is defined at randomization with the objective to assess whether the effectiveness of dexamethasone added to TBM treatment is genotype dependent [369]. A possible outcome of the trial is that it may be possible to use *lta4h* genotyping to target corticosteroid therapy to patients most likely to benefit from it. Further genetic studies in TB-IRIS could identify genes that may also identify patients that benefit most from interventions such as prophylactic prednisone. In our study cohort, I have not been able to show *lta4h* genotype can successfully identify these patients.

The clinical diagnosis of TB-IRIS remains challenging. Its most common presentations are lymphadenopathy; respiratory symptoms like worsening cough, chest pain, or worsening chest X-ray features; and constitutional symptoms like night sweats, fever, loss of appetite, and loss of weight. Abscesses or neurological features are less common [18]. Moreover, less specific symptoms like vomiting, diarrhea, arthritis or back pain may all be presenting symptoms of TB-IRIS. It is difficult to distinguish these signs and symptoms from other causes for deterioration like other opportunistic infections, drug toxicity, drug resistance or non-adherence. Many studies have been done, trying to identify biomarkers that can aid identification of TB-IRIS [21, 38, 40, 48, 49, 54, 59-66, 69, 70, 146, 151, 370-372]. Most of these studies, however, include tests that are not readily available in those parts of the world where TB and HIV are most prevalent. Clinicians treating patients with HIV-associated TB would benefit from easily accessible laboratory tests that could aid in diagnosing TB-IRIS. CRP is becoming available as a point-of-care assay [135]. In our study, CRP looks like a promising tool to rule out TB-IRIS: a low CRP appears useful in excluding TB-IRIS, but higher cut-off values of CRP still have value in ruling it out. Further studies are needed to confirm this finding.

Lastly, lung function impairment needs to be taken into consideration as part of the treatment of patients with HIV-associated TB. Post-tuberculosis lung disease is also common in patients co-infected with HIV, and recently more data on this topic became available. A study from Zimbabwe assessed chronic lung disease in patients who completed TB re-treatment on average two years prior; 65% were co-infected with HIV. Chronic lung disease - defined as a combination of chest X-ray abnormalities, respiratory symptoms and abnormal spirometry and/or walk test and /or desaturation at exertion - was present in 14% of the participants [373]. A study from South Africa, prospectively following patients with

HIV-associated TB who started treatment for both, found spirometric lung function impairment in 33% of participants at 12 months [374]. A prospective cohort study from Malawi found HIV-uninfected patients were more likely to suffer from post-tuberculosis lung disease. However, of participants with HIV-associated TB, 25% had abnormal spirometry results 12 months after completing treatment for TB [375]. In a prospective study cohort form Mozambique – with 63% of the participants co-infected with HIV – abnormal spirometry outcomes were present in 65% of patients one year after start of TB treatment [376]. In all these studies, lung function impairment was not limited to one type, but could be obstructive, restrictive or both. I showed abnormal spirometry results in 53% of our study participants at completion of TB treatment. Important for clinical practice is that chest X-rays were insufficient to detect lung function abnormalities in our patients with HIV-associated tuberculosis and a low CD4 count.

With a considerable proportion of patients with newly diagnosed HIV still presenting late for care [377-379] and TB incidence worldwide only declining slowly [11], TB-IRIS remains an important health topic. Multiple studies in the past twenty years have contributed to our knowledge of this syndrome, its incidence and risk factors; diagnosis, prevention, management and outcomes; and its pathogenesis. However, many of its aspects remain incompletely understood. In this thesis I investigated several aspects of TB-IRIS, focusing on the role of prednisone in the immunopathogenesis of TB-IRIS and its effect on lung function outcomes. I was able to confirm recent findings that an interaction between the innate and the adaptive immune system plays an important role in TB-IRIS [60, 64] and showed that prednisone prophylaxis was associated with a reduction in plasma concentrations of the chemo- and cytokines found to be increased in TB-IRIS. I assessed the effect of *lta4h* genotype and was unable to show an association between *lta4h* genotype and either the concentrations of chemo- and cytokines or the efficacy of prednisone. Prednisone as used in our trial was associated with better lung function 4 weeks after starting ART but did not affect long-term lung function outcomes in our study cohort with a high prevalence of TB-IRIS. These findings can guide researchers investigating further mechanisms and ways to prevent TB-IRIS or improve overall outcomes in HIV-associated TB.

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