Published in final edited form as:
Int J Tuberc Lung Dis. 2009 February ; 13(2): 153–164.

What has "Karonga" taught us? Tuberculosis studied over three decades

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Abstract

This paper summarises tuberculosis (TB) research over almost 30 years in Karonga District, northern Malawi, an area typical of much of rural Africa. The dominant factor has been HIV, which arrived in the district about 1980, leading to an increase in TB incidence to a peak of approximately 65 smear-positive pulmonary cases/100,000 in 2000. Tuberculin surveys indicate annual risks of *M. tuberculosis* infection of approximately 1%, thus most of the population is uninfected and at risk of primary infection and disease. Molecular epidemiological studies demonstrate that about two thirds of TB arises from recent infection, but recognisable recent contact is responsible for only about 10 % of disease. By 2001, 57% of TB was directly attributable to HIV, implying that it would have declined were it not for HIV. HIV infection increases the risk of TB most among young adults, and greatly increases the risk of recurrence from new infection after treatment. Mortality rates in the HIV-infected are high, but there is no association of HIV with drug resistance. Other risk factors with relatively smaller effects include age and sex, contact, several genetic polymorphisms and area.

Neither one nor two doses of BCG provides protection against adult pulmonary tuberculosis, despite protecting against leprosy. Skin test surveys, cohort studies and comparative immunological studies with the UK suggest that exposure to environmental mycobacteria provides some protection against TB and that BCG's failure is attributable partly to this widespread heterologous exposure masking effects of the vaccine.

Drug resistance has remained constant (<10%) over more than 20 years. Immunotherapy with *Mycobacterium vaccae* provided no benefit, but treatment of HIV-positive patients with cotrimoxazole reduced mortality. The Karonga programme illustrates the value of long-term population-based studies to investigate the natural history of TB and to influence TB control policy. Current studies focus on immunological markers of infection, disease and protection, and on elucidating the impact of anti-retroviral therapy on TB incidence at population level.

Introduction

A large research project on the epidemiology of mycobacterial infection and disease was initiated in Karonga, a district in northern Malawi, in 1979. The programme's focus evolved over subsequent years, tracing a decline in leprosy, the emergence of HIV, and a great increase in tuberculosis. The work has included classical and molecular epidemiology, vaccine and treatment trials, as well as demography, immunology, and genetics. This paper describes the contribution of these studies to our understanding of tuberculosis, from natural history to control, including interactions with HIV, and immunological interactions with

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other mycobacterial infections. It illustrates the value of long-term community-wide studies for understanding a complex disease in a population context.

The overall programme, known as the Karonga Prevention Study (KPS) has also made significant contributions in other areas, particularly in leprosy and HIV/AIDS. A full publication list is available on www.lshtm.ac.uk/ideu/kps

Setting and methods

Karonga District is bordered to the east by Lake Malawi and to the west by the Central African (Nyika) Plateau (Figure 1). The population consists primarily of subsistence farmers, fishermen and small traders, and has grown from approximately 112,000 to over 250,000 over the past 30 years. The area was selected for its high prevalence of leprosy and its isolated situation, a factor associated with relatively little population movement. Two total population surveys were carried out in 1979–1984 and 1986–1989. [1,2] These surveys developed methods for precise grid mapping (later translated to GIS coordinates) and for accurate identification of individuals seen repeatedly, in different circumstances, over time. These and other methods have been continued ever since, enabling linking of interview data, clinical examinations and biological samples within and between studies. The linked databases now contain data from 800,000 contacts with 300,000 individuals.

KPS staff are stationed at all the major health facilities, where they screen inpatients and outpatients for symptoms of tuberculosis. Additionally, everyone participating in community-based studies is asked about chronic cough. Surveys have confirmed that the district-wide tuberculosis case detection rate is high. The KPS laboratory has processed all biological samples from district tuberculosis suspects since late 1985, undertaking both sputum smear microscopy and culture. Isolates have been sent to the UK for species confirmation, drug sensitivities and molecular typing. The KPS works closely with the Malawi National TB Programme and acts on its behalf in Karonga District in co-operation with the District Tuberculosis Officer, enhancing the care for tuberculosis patients in the district. The numbers of diagnosed tuberculosis cases increased greatly over the history of the KPS. The incidence of confirmed smear-positive pulmonary disease peaked in the mid-late 1990's at around 130/100,000 adults per annum (this represents approximately 65/100,000 total population), and then declined to its current level of below 80/100,000 adults[3].

HIV data are available on the Karonga population in various contexts: clinical settings; large, ongoing case-control studies; antenatal clinic surveillance and house-to-house surveys. The earliest infections were identified from archived specimens collected in 1982[4], and by 1988 HIV testing procedures had been established at the site. HIV prevalence was 3.9% of the 15-49 year-old population by 1988-1990, increased to 11% in 1991-1993 and reached a plateau of around 13% in 1998-2001[5,6].

BCG vaccination was introduced in Karonga district in mass school campaigns in the mid 1970s. Since 1990 it has been given at first health system contact, as part of the Expanded Programme on Immunization.

Epidemiology of *M. tuberculosis* infection

The patterns of incidence and prevalence of *M.tuberculosis* infection in the Karonga population, have been described in detail, through analysis of population-based tuberculin survey data conducted with consistent and rigorous protocols[7] (using RT23 tuberculin (2IU) throughout). More than 63,000 individuals were tested in the first total population survey (1979–1884) and more than 45,000 in the second (1986–1989), making this the largest and

most detailed dataset on tuberculin skin tests collected in Africa[7,8]. Seven thousand individuals were tested in both of the surveys and in addition approximately 9,000 tuberculin tests have been carried out from 1996-2008 in the context of studies of the household transmission of *M.tuberculosis* and of the immunogenicity of BCG and other vaccines[3,9].

Figure 2 shows the pattern of delayed-type hypersensitivity (DTH) to tuberculin among females without a BCG scar, by age, in the early 1980s and Figure 3 shows the proportion with indurations above 10 mm, by age, sex and BCG scar status[7]. These patterns are typical of many populations[10,11], the increase by age reflecting cumulative lifetime exposure and the plateau and decline after age 50 reflecting ageing of the immune system. Trends are similar between males and females until approximately 15 years of age; males show consistently higher prevalence above this age. This too is typical of many populations[10,11], though less understood: it may reflect in part a greater likelihood of exposure among adult males compared to females, but may also reflect a sex-difference in immune response to infection – males generating a stronger and/or more durable DTH response to *M.tuberculosis*, compared to females.

Such data are often used to derive annual risks of *M.tuberculosis* infection, though such inferences are more complex than widely appreciated[7,12]. Analyses of the KPS age-specific prevalence data by conventional methods provided estimates of annual rates of infection ranging from approximately 0.5 to 1.1 percent per annum, depending upon the criterion used for tuberculin “positivity” (for example a simple 10 mm criterion, or assuming a symmetric distribution of true positive responses around 17 mm, ie the “mirror method”)[7,13] Mixture analysis methods which attempt to distinguish sensitivity attributable to *M.tuberculosis* from that attributable to exposure to other mycobacteria[14], suggest prevalence (thus incidence) estimates intermediate between the other methods.

The 7,000 individuals tested in both KPS surveys allowed longitudinal estimation of tuberculin conversion and reversion rates in individuals[7]. A change from below to above 10 mm with an absolute increase of at least 6 mm was defined as conversion[15]. Reversion was defined in equivalent but opposite terms. Amongst individuals without a BCG scar, conversion rates increased and reversion rates decreased with age, as observed elsewhere[16-19] . Analysis of Karonga data indicates that such apparent trends may be artefacts due to instability of the tuberculin test, being what would be expected if infection risks were actually constant by age. The proportion of test negatives which are false (due to problems with the test) will rise with age, as the true prevalence of positivity increases with age. As positive individuals who were falsely negative on an initial test will appear to have converted if their second test is (correctly) positive, this will produce an apparent increase in conversion risk with age. The reverse logic applies to reversion rates - in young infants a high proportion of initial “positive” tests will be false and with a subsequent negative test, will be interpreted as reversion. Many authors have commented on the instability of tuberculin testing (eg it is well known that exposure to environmental mycobacteria induces tuberculin DTH, that induced DTH to BCG rises and then falls after exposure[20]; and that some virus infections induce temporary suppression of DTH) but these KPS analyses provide an unusually detailed insight into this instability and have important implications for estimates of infection risks.

Analysis of tuberculin tests carried out on 2,400 individuals in the months after BCG vaccination further our understanding of DTH responses, showing that it rises to a peak 1 to 3 months after BCG exposure, with almost all individuals making a response at this time, and then declines[20] (Figure 4). Whether an equivalent trend occurs after *M tuberculosis* infection is unclear, as the decline may reflect the disappearance of BCG bacilli.

Comparisons of tuberculin reactivity with whole blood *in vitro* assays of interferon gamma release after exposure to *M.tuberculosis* RT48 antigen reveal moderately strong positive correlations[9], but with notable exceptions: a small proportion of individuals with strong DTH show no IFNg release, and a small proportion with no measurable DTH release large quantities of IFNg on exposure to similar *M.tuberculosis* antigens. Other researchers have found analogous inconsistencies between skin test and in vitro responses to mycobacterial antigens[21,22], but the implications for inferring infection and protective immunity are unclear.

The estimate of around 1% infection risk in Karonga is similar to that for many high burden countries today, and to Europe in the mid 20th century (after declining from much higher levels). The highest infection risks today appear to be 3 %- 4% in the Western Cape of South Africa.

Importantly, this estimate means that, even were infection life-long, the large majority of the population of Karonga (median age approximately 15) at any given time are not infected with *M.tuberculosis*. Most individuals in such a population are thus at risk of primary infection and primary tuberculosis disease.

Recent versus latent infection in disease

There has been a long-standing debate over the relative importance of reactivation of past infection versus recent infection or reinfection as causes of tuberculosis disease in adults. The development of IS6110 based molecular fingerprinting[23] has provided a tool for investigating this issue, by distinguishing “strains” of *M tuberculosis*. The sharing of identical strains by cases suggests recent transmission rather than reactivation of old infection, and so the extent of “clustering” (ie the proportion of cases who have a strain common with at least one other case) in a population can be used to infer the extent of recent transmission[24]. These studies are most informative when, as in Karonga, they are conducted in whole populations over several years[25].

Molecular fingerprinting data are available at KPS from 1996. The proportion of cases who had a strain common to another case within the study period (ie were “clustered”) was 72%, among the highest recorded in any setting[26]. Using our longitudinal data, we calculated the proportion of cases clustering with cases diagnosed one to eight years previously. The maximum proportion clustered was reached within a 4-year time window, indicating that approximately two thirds of cases were due to recent transmission. This appeared to be stable over recent years.[26] Analysis of clustering by time windows avoids underestimation due to incomplete inclusion of clusters in studies with shorter time frames. Having a strain in common with other cases became less likely with increasing age of HIV-negative patients as expected, since the proportion of reactivation disease will increase with age[26].

Role of recent *known* contact with smear positive cases

One corollary of a high proportion of cases arising from recent infection is that contact tracing might be useful in tuberculosis control, with an effect which is measurable in the short-term. However, investigation of same-strain clusters in Karonga revealed only 11% of clustered cases had an identifiable epidemiological link within their cluster[27]. We asked all confirmed tuberculosis patients about known prior contacts with tuberculosis, and also identified contacts from the project database[27]. Transmission from identified smear-positive putative source contacts was only confirmed for 44% of family and household contacts, and a lower percentage for other contacts. Some cases will have arisen from reactivation of latent infection (non-clustered), but the implication is that even tuberculosis patients with disease arising from recent infection who have recent smear-positive cases in their family or

household, may well have acquired their infection elsewhere. Mixed strain infection could account for some of the differences although even in a high incidence area, they are thought to be unusual[28].

Using the proportion of epidemiologically-linked cases for whom transmission was confirmed and the prevalence of household or family contacts, we estimated that 9-13% of all cases were due to recent transmission in the family or household[27]. Using an alternative, case-control approach we confirmed that identifiable recent contact with known smear-positive cases accounted for only 12.5% of the tuberculosis burden, nearly half of that (5%) attributable to having nursed, or shared a bedroom with, a relative with smear-positive pulmonary tuberculosis[29]. The low proportion of tuberculosis attributable to identifiable links is consistent with findings from South Africa and India (using different methods)[30-32]. Although the risk of infection in household contacts of smear-positive tuberculosis cases in these and similar settings is greatly increased[33] compared to those without such contacts, all our findings are consistent with the large majority of *M.tuberculosis* transmission in this setting arising from casual contact.

Role of different strains

Investigation of the clusters also identified particular strains and strain families, some of which are common worldwide. In Karonga the Beijing genotype accounted for 4.3% of strains. It appears to have increased over time, and was fully drug sensitive[34].

Impact of HIV on rates of disease, recurrence and transmission

Over the history of the KPS, by far the strongest risk factor for tuberculosis in Malawi, as elsewhere, has been HIV. Although the effect of HIV on risk of *M.tuberculosis* infection is unknown, HIV infection increases the risk of disease following both latent and recent infection, but not necessarily to the same extent, and HIV also affects the course of disease and the infectiousness of cases. Although these patterns have been investigated in many contexts[35], the KPS incorporates the only long-term population-based molecular epidemiological study of tuberculosis in an area with a high prevalence of HIV. It has therefore been uniquely placed to assess the effect of HIV on different mechanisms of disease.

A case-control study in Karonga in 1988-1989 found that the odds ratio for the association of tuberculosis with HIV infection was 7.4 (95% ci 3.3-16.7%)[36], at which time 28% of confirmed tuberculosis patients were HIV-positive[37]. A cohort study gave similar results, recording tuberculosis incidence up to 1996 in 11,000 individuals with known HIV status seen in the 1986-89 survey, and finding a relative risk of 7.1 (95% ci 3.2-15.7)[38] In a case control study among tuberculosis cases diagnosed in 1996-2001 the odds ratio for the association with HIV infection had increased to 12.0 (9.0-16.1). These results reflect the increasing proportion of prevalent HIV-infected individuals with more advanced immunosuppression as the HIV epidemic progresses. By this time 64% of confirmed tuberculosis patients were HIV-positive[39] and this proportion has remained constant.

Estimates of the proportion of smear-positive tuberculosis cases directly attributable to HIV increased from 17% in 1988-1990 to 57% in 2000-2001. These estimates do not account for increase due to onward transmission from HIV-positive cases. We estimated that over the same period the rate of smear-positive tuberculosis in adults would have decreased from 0.78/1000 to 0.45/1000 in the absence of HIV, and by 2003-2005 to 0.35/1000[37] (Figure 5).

There is some evidence that HIV increases the risk of disease following recent infection more than that due to reactivation. It would be expected that the relative risk for the

association of HIV and TB would increase with age, reflecting increasing immunosuppression in those infected longer with HIV. However in Karonga[39] (Figure 6) and elsewhere[40] the relative risk of tuberculosis in those with HIV infection is higher in younger adults than in older adults. Since younger adults are less likely than older adults to have reactivation disease, this is consistent with HIV infection having a greater effect on disease following recent infection.

An association of HIV with cases having strains in common (clustered) might suggest that HIV has a greater influence on disease following recent infection than on disease following past infection, although this is complicated by lower infectiousness of HIV-positive patients and by the difficulty of establishing the sequence of transmission within a cluster. In fact, HIV infection has been associated with clustering in some studies but not in others[24,30,41]. Where HIV and tuberculosis are only present in subgroups of the population, an association with clustering may represent increased opportunity for transmission (eg in nosocomial settings) and thus not reflect any differences in mechanism of disease. Only a few, mostly small, studies are in populations with generalised HIV epidemics and the results are variable[30,41-47]. In Karonga, HIV infection is associated with clustering in adults aged 45 years or older but not in younger age groups (in which the proportion clustered was already very high)[26] with no association between HIV and cluster size[48].

HIV-positive tuberculosis patients have high mortality rates. In our setting, for patients with smear-positive pulmonary tuberculosis, HIV increased the mortality of smear-positive pulmonary tuberculosis 6-fold during treatment, and 8-fold thereafter[49], although it did not influence the smear conversion rate. Mortality rates both during and after treatment depend on the patient population. In a comparison of case fatality rates in Karonga and Lusaka (Zambia) among HIV-positive tuberculosis patients using data from the *M vaccae* trial (see below), higher rates found in Karonga could be almost entirely explained by patients being older and with more advanced HIV disease[50].

Using molecular fingerprinting, rates of recurrence due to relapse and reinfection were calculated over eight years. Similar rates of relapse were found in HIV-negative and HIV-positive patients, but disease due to reinfection was found to be rare in HIV-negative patients (0.35 /100 person years)[51], though as common as relapse in HIV-positive patients (2.23/100py). This was the first population-based study to estimate rates of relapse and reinfection by HIV status.

Several studies have investigated the relative infectivity of HIV-positive tuberculosis patients by examining tuberculin skin test conversion in household contacts. In general these have suggested lower transmission from HIV-positive than from HIV negative cases[52-54], which might reflect either different pathology or earlier treatment if HIV-infected patients sicken and seek care more quickly. In the KPS, we examined HIV status of smear-positive index cases and compared *M.tuberculosis* strains with cases who named the index case as a putative source. We found that if the index case was HIV-positive they were only half as likely as HIV-negative index cases to be confirmed as the source of infection by molecular fingerprinting[27]. In addition, HIV-positive tuberculosis patients were less likely than HIV-negative patients to be identified as prior contacts of subsequent tuberculosis cases even when adjusted for smear-positivity and closeness of contact.[29] Nevertheless, despite the lower transmission, because approximately two thirds of smear-positive patients in the area are HIV-positive, we estimate that nearly half of the transmission of *M.tuberculosis* in this setting is from HIV-positive patients[27].

Effects of gender

In Karonga, as elsewhere there is a distinct difference in tuberculosis patterns by sex (Figure 7), with excesses in younger women and older men[37,39]. The excess in younger women is largely attributable to the higher HIV prevalence in women in these age groups. Recent close contact with tuberculosis patients may also explain some of this gender distribution[29,39], as young women in this community preferentially perform the nursing roles within the family. Women were more likely than men to have strains in common with other patients[26]. However among those with clustered strains, being part of a large cluster was more frequent in men, in younger adults and in the urban area[48]. The sex pattern is consistent with women being more likely to be infected within the home (therefore being identified as clustered within the study population, but in small clusters) and men being more likely to be infected outside their homes (therefore either in large clusters, or not clustered if infected outside the study area)[48].

Adult women are less likely than adult men to have a “positive” tuberculin skin test, regardless of the criterion used (Figure 3) [7] This may reflect a different immune response to earlier infection or a lack of exposure, due to social patterns changing after childhood. Lack of prior infection may leave young adult women more susceptible than men to primary infection and primary disease. Case-control studies in Karonga found no effect of cooking-smoke exposure or pregnancy on the risk of tuberculosis in women[39].

Diagnosis

KPS has followed standard diagnostic algorithms and procedures for identifying pulmonary tuberculosis cases, using smear microscopy and culture of biological samples from tuberculosis suspects in a well-functioning laboratory with rigorous quality control. In the Karonga setting it was found that 97% of those who were smear-positive on three smears could have been identified with only two smears, with similar sensitivity and specificity, using culture as gold standard, and that this was not affected by the HIV status of the tuberculosis patient[55]. This has contributed to recommendations that overstretched TB control programmes with appropriate laboratory quality control may safely reduce the numbers of sputum smears in screening[56]. In addition, a diagnostic clinical scoring system (validated by fine needle aspirate or histology) was developed to distinguish tuberculous lymphadenitis from lymphadenopathy of other causes (primarily HIV-associated) in a setting where HIV had greatly increased the incidence of lymphadenopathy of many causes[57].

Other operational studies from Karonga have demonstrated that individuals with less certain diagnoses of tuberculosis (ie lack of culture confirmation, or positive results from a single specimen only) are at higher risk of mortality[49], independent of HIV status, presumably due to other (untreated) pathology. A high proportion of chronic cough suspects in this setting who are not diagnosed with tuberculosis are HIV-positive and exhibit clinical syndromes consistent with WHO Stage 3 or Stage 4 AIDS[58] and should be referred directly to antiretroviral therapy (ART) services.

Treatment

The KPS follows standard Malawi treatment protocols for tuberculosis, and thus is well-placed to provide reliable, consistent long-term data on the prevalence of drug-resistance in a rural African population with a moderate HIV prevalence and a well-functioning tuberculosis control programme. To date, second-line drugs are not widely available. Initial drug resistance to one or more drugs has consistently been around 10% and multi-drug resistance is rare, at less than 1%, with no increase over the past 20 years [59-61].

In other analyses of routine KPS data, it was established that reported treatment outcome is misleading if based solely on registered patients, since diagnosed patients who die or default before registration are excluded. In Karonga case fatality rates were 16% among all diagnosed smear-positive patients, compared to 13% of those registered for treatment[49]. This observation led to a call for National Tuberculosis Programmes to use diagnosed rather than registered patients as the denominator for treatment outcome statistics[62].

Karonga has participated in two multicentre studies to evaluate enhancement of tuberculosis treatment. A randomised placebo-controlled trial demonstrated that immunotherapy with single-dose *M.vaccae* has no place as an adjunct to standard tuberculosis treatment in HIV-positive adults[63]. An open trial with a historical comparison cohort showed that even in a country where co-trimoxazole is standard first line treatment for several diseases in adults and children, and where sulfadoxine-pyrimethamine was first line treatment for malaria (thereby making the presence of co-trimoxazole resistant organisms more likely), a prophylactic 12 month course of co-trimoxazole in HIV-positive tuberculosis patients had a significant impact on mortality, averting one death during the 18 months post-registration for every 12.5 HIV-positive tuberculosis patients treated[64]. The results of this study were a major factor leading to country-wide adoption of co-trimoxazole into standard care for all HIV-positive tuberculosis patients in Malawi.

Genetic susceptibility

Genetic susceptibility to tuberculosis and leprosy has been studied using multi-case family trees that can be drawn up from Karonga databases, and in large multi-centre case-control studies. These studies have contributed to methodological work on family sampling in genetics studies[65], and found that several genetic associations with tuberculosis seen in other populations (variants of vitamin D receptor, interferon-γ, mannose-binding lectin, and Solute Carrier family 11 [SLC11A1, formerly NRAMP1]) could not be replicated in this population. Other polymorphisms appear to be associated with tuberculosis in Karonga: for example an untranslated region of SLC11A1 (associated with protection) and Q1022H in Complement Receptor 1 (associated with susceptibility)[66]. Some associations found in Karonga differed by HIV status, suggesting that the role of innate immune responses may be materially altered in the immunocompromised host[66]. Further analyses as part of a multi-centre study have suggested a protective effect of the CD209-336G variant allele of the DC_SIGN receptor[67] and work is ongoing.

BCG

BCG has been studied intensively in Karonga, in terms of efficacy, immune responses, the interpretation of BCG scars and the effect of BCG on subsequent DTH to tuberculin. Two large studies of BCG efficacy were conducted, the first a cohort study based on over 80,000 individuals, defined by BCG scar status[68] (many of whom had been vaccinated in mass campaigns carried out in schoolchildren), and the second a randomised controlled trial of single versus repeat BCG in over 120,000 individuals, with a 5 to 9 year follow-up[69]. The cohort study established that although BCG provided 50% protection against leprosy, no protection against tuberculosis was demonstrable in this population (and scar size did not correlate with protection against either disease[70]). The trial confirmed that the lack of protection against tuberculosis was not overcome by repeating the vaccination in adults, although the protection against leprosy was improved[69]. This is one of the results underlying WHO's recommendation against giving BCG boosters.

The findings in the Karonga studies were important in that they established that the failure of BCG in this population was a specific deficit in BCG's action against tuberculosis: effects against leprosy were clear cut and supported continuation of the BCG programme in the

efforts to eliminate leprosy. The findings related to leprosy also allayed concerns over methodological issues such as attribution of vaccine status by scar observation in the cohort study. It is known, from a district-wide blinded study in Karonga, that less than 60% of children vaccinated as infants have a recognisable scar 3 years later, and that scar reading is subject to considerable observer variation particularly in young children[71]. Even BCG given in the controlled circumstances of the efficacy trial failed to produce a scar in approximately 7% of individuals aged between 3 months and 60 years at the time of vaccination[8], and more than 20% of infants vaccinated before one month of age did not have a recognisable scar at 4 years of age[8]. This has implications not only for efficacy studies, but also for vaccine uptake surveys[8,71].

The need to elucidate the relationship between reactogenicity (ability to induce scars and ulcers), immunogenicity (ability to induce DTH or cytokines) and protection (afforded against tuberculosis and leprosy) in the response to BCG vaccination [72] and the different actions of BCG in different settings, resulted in further studies of BCG in Karonga, and in parallel studies in the UK (in the UK, adolescent BCG vaccination has been shown to be protective against pulmonary tuberculosis).

Efficacy of BCG in stimulating immune response and difference between populations

Placebo controlled studies of immune responses to BCG amongst young adults and adolesents in Karonga and in the UK used a diluted whole blood assay[73] in which cytokines produced by monocytes could be measured in 24-hour culture supernatants by ELISA[74] or IFN-γ and other T cell derived cytokines could be measured after 6 days incubation[9]. Using these assays, Malawian subjects were shown to produce higher amounts of proinflammatory cytokines such as TNF-α, IL-1β and IL-10 than UK subjects[74]. T cell responses to *M.tuberculosis* PPD and several other mycobacterial antigens were assessed in terms of IFN-γ production before and one year after BCG vaccination of young adults and adolescents in Malawi and the UK. A much higher proportion of Malawian than UK subjects were IFN-γ responders prior to vaccination whereas this proportion was similar (approximately 80%) between the two populations one year after vaccination[75]. This difference probably reflects more extensive prior exposure to environmental mycobacteria[76] in Malawi compared to the UK [77], although we were unable to demonstrate an association between prior *in vitro* sensitivity to antigens from particular environmental mycobacteria in the UK and failure to increase IFN-γ production following vaccination[78,79]. Using an assay to detect clonal T cell expansions, new T cell clones were detected in the Malawians following vaccination[80], but such immunity may be lost over time following exposure to the many other infections in a rural African setting. Studies in BCG vaccinated infants in Malawi and the UK, showed that there are differences in how infants respond to vaccination in the two settings[3], suggesting that response is influenced by factors other than life-time exposure to environmental mycobacteria. These comparative studies highlight that the immune system is probably configured and maintained differently in an African compared to a European setting, with implications for both the induction and maintenance of vaccine-induced immunity.

Environmental mycobacteria: their role in *M.tuberculosis* infection, disease and immune response

Environmental mycobacteria have been an important theme in KPS studies: they induce non-specific tuberculin reactivity and so must be considered in interpreting tuberculin surveys and *in vitro* immunological assays with mycobacterial antigens; they contaminate

sputum specimens and appear as acid fast bacilli in sputum smears, necessitating culture for definitive diagnoses of tuberculosis; and exposure to them may influence the effectiveness of BCG vaccination.

Several studies have shown a widespread and heterogeneous environmental mycobacterial flora in Karonga. Examination of soil and water samples by culture, acid fast staining and PCR, or by direct PCR using 16S rRNA revealed evidence for ubiquitous mycobacteria[81]. Several isolates were members of the *M fortuitum* complex, but speciation proved difficult, and it is likely many of the local mycobacteria have not been characterised. Over 13 years, 474 non-tuberculous mycobacteria were identified in Karonga sputum cultures by our UK reference laboratory. More than half were in the *M avium-intracellulare* complex, and 25 % were *M fortuitum*, with a wide range of less common species[82], all adding to the evidence for a rich mycobacterial flora in contact with humans.

On a larger scale, more than 36,000 individuals were skin tested during the early 1980s, with 15 antigens from 12 environmental mycobacteria[82]. Detailed analyses of these results by age, sex and BCG scar status revealed considerable variation in the frequency of sensitivity to these antigens. The highest prevalence was noted to antigens of *M avium, intracellulare* and *scrofulaceum (*"MAIS" complex*)*, among the slow grower group, and to one of three *M fortuitum* antigens among the fast growers (corresponding to frequencies found in sputa). Sensitivity to antigens of several of the slow-growers (*M avium*, *M intracellulare*, *M kansasii*, *M marinum*, and *M scrofulaceum*) was increased significantly in individuals with a BCG scar, reflecting cross reactivity of these organisms, all of which are known to be close relatives of *M.tuberculosis*. In analysis of subsequent tuberculosis and leprosy incidence, prior sensitivity to antigens of fast growers (eg *M fortuitum*) but not to antigens of slow growers, was associated with 70 % lower incidence of both tuberculosis ($p = 0.08$) and leprosy ($p = 0.05$). This was unexpected, as we had predicted that sensitivity to slow growers would be most strongly associated with reduced incidence of tuberculosis and leprosy as found elsewhere[83], given that *M.tuberculosis* and *M leprae* are classified with the slow growers. The agreement between the effects on tuberculosis and leprosy supports the validity of the KPS results, but the mechanism is unclear. It may reflect particular antigenic properties of the mycobacteria prevalent in the Karonga environment, and the observation is also consistent with hypotheses that "specific" DTH responses (ie to closely related antigens) are associated more with immunopathology than with protection[84,85].

Related to these studies, six isolates of three species of non-tuberculous mycobacteria from Karonga (*M avium*, *M cheloni*, *M fortuitum*) were included in collaborative experiments on mycobacterial vaccines in mice[86]. Only the *M avium* strains replicated in mice, and prior exposure to *M avium* alone inhibited replication of BCG bacilli given subsequently. This suggests *blocking* of BCG's action by prior environmental mycobacterial exposure, rather than *masking* of BCG's effect as originally proposed and demonstrated[87]. It might explain why BCG given to adults failed to protect against tuberculosis in Karonga, although it does not explain why BCG protected against leprosy in the same trial (although a relatively weak immune response may be sufficient to inhibit *M leprae*). Given that BCG vaccination is now given only to young infants, too early for prior environmental mycobacterial infection, we still suspect that the influence of these other mycobacterial infections is more in masking than in blocking of BCG.

Summary

The Karonga programme has allowed exploration of many aspects of the interaction between *M.tuberculosis* and the human host in a single population: from genetic predisposition to infection and disease, through risks of infection, development of disease,

disease trends, the influence of HIV and environmental mycobacteria and the complex action of BCG vaccine. The combination of all these studies in a rigorous, broad, and long-term framework puts each of the separate findings in context, making them more interpretable, and generalisable. They highlight the complex nature of the relationship of *M.tuberculosis* (and *M.leprae*) with the immune system of the host. With no other pathogen is there evidence that incidental exposures to related species have such a profound effect on risk, on response to vaccines and on interpretation of measures of infection and protection. The relationship between HIV and tuberculosis is also special, having an evolving synergistic relationship that exacerbates the severity of each. Tuberculosis has always been an opportunist and HIV has facilitated its re-mergence as described in Karonga and throughout the world. In addition to the basic science research carried out by the KPS, the relationship with the NTP has facilitated operational investigations into drug resistance, treatment outcome, co-trimoxazole preventive therapy and optimal diagnostic use of microscopy services.

Future of tuberculosis and tuberculosis research in Karonga district

The KPS continues its epidemiological and immunological research on tuberculosis, including participation in multicentre studies exploring markers of *M.tuberculosis* infection, disease and protection. The advent of ART has already been shown to have a measurable effect on mortality at population level in the district[88] and it is likely that the epidemiology of tuberculosis will change. The effect is unpredictable - gains made may be counterbalanced by the increasing survival of a large cohort of chronically immunosuppressed individuals at moderate to high risk of tuberculosis and the emergence of tuberculosis associated with immune-reconstitution disease. The KPS involvement with tuberculosis control, HIV surveillance and ART delivery in Karonga district is ongoing in both a research and operational capacity. Current and future studies should make an important contribution to the identification of new challenges, and appropriate responses in this new era of tuberculosis in sub-Saharan Africa.

Acknowledgments

The authors are grateful to to Hazel Dockrell and Sian Floyd for contributing to and commenting on this review. Also to past Field Directors, Assistant Field Directors and Key Investigators: Jorg Ponnighaus, Susan Oxborrow, David Warndorff, Bagrey Ngwira, Frank Mwaungulu, Nuala McGrath, Neil French (current programme director), Hazel Dockrell and Basia Zaba. And the hundreds of people who have worked on the programme, particularly Luckson Mwanjasi, Simon Malema, Peter Gruer, Cathy Gruer, Lyn Bliss, Lifted Sichali, Venance Mwinuka, Glyn Msiska, Steve Chaguluka, Jacky Saul, Sian Floyd, Keith Branson. Also to collaborators in the Malawi National Tuberculosis Programme and the Ministry of Health, and many from other institutions, both in Malawi and overseas, including the Community Health Sciences Unit, Malawi; the London School of Hygiene and Tropical Medicine, UK, the Health Protection Agency, UK; and the Wellcome Trust Centre for Human Genetics, UK. And to the several funding bodies which have supported the work, in particular LEPRA (The British Leprosy Relief Association), the World Health Organisation and the Wellcome Trust.

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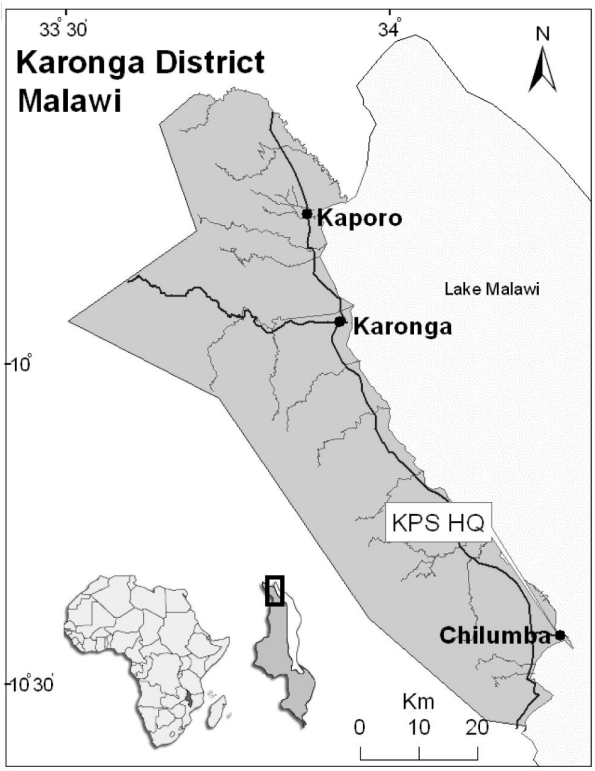

Figure 1.
Map of Karonga

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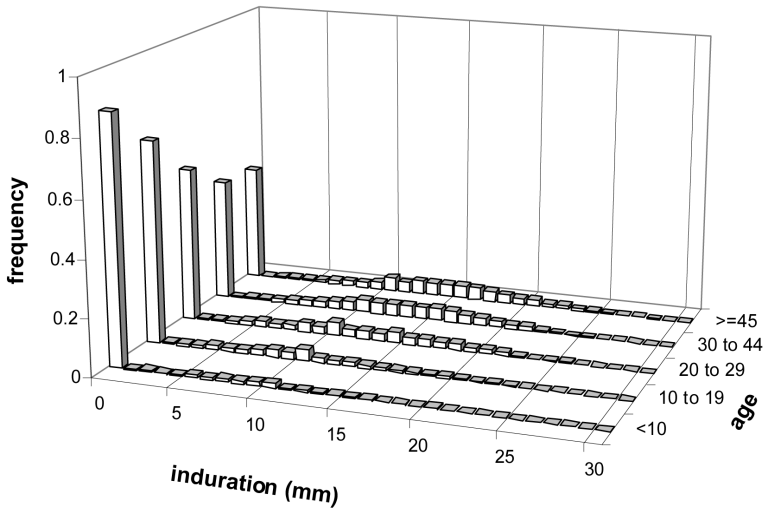

Figure 2.
Frequency distributions of induration to RT23 in females with no BCG scar by age, Karonga district 1980-1984

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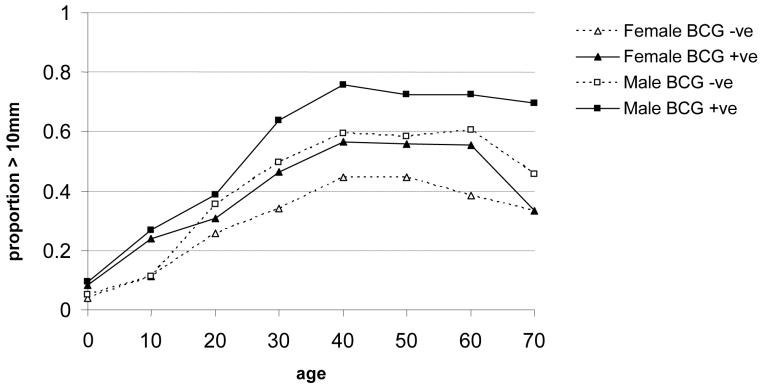

Figure 3.
Prevalence of induration greater than 10 mm, by age, sex and BCG scar status, Karonga 1980-1984.

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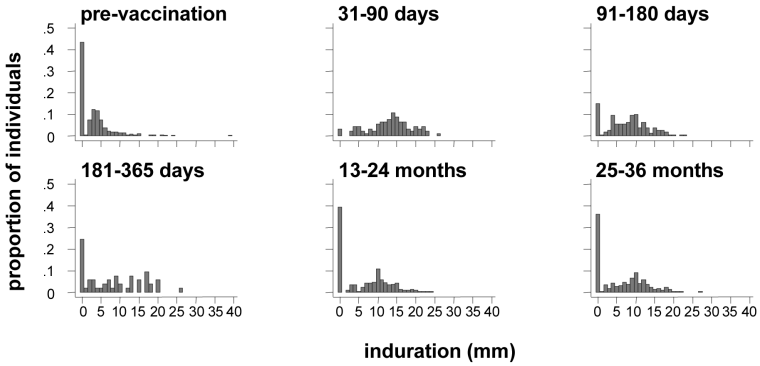

Figure 4.
DTH response to tuberculin and time since vaccination, among individuals aged <15 who were BCG scar negative at vaccination.

Figure 5.
Rate of smear-positive pulmonary tuberculosis in adults aged 15-49, Karonga, 1998-2005.

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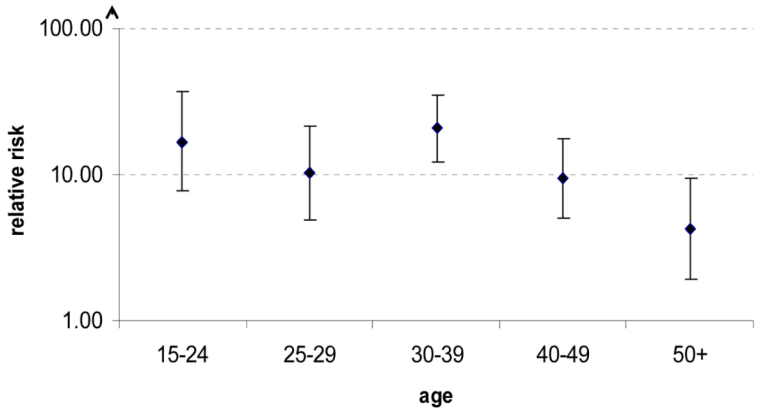

Figure 6.
Relative risk of TB in HIV-positive vs HIV-negative adults in northern Malawi, by age group

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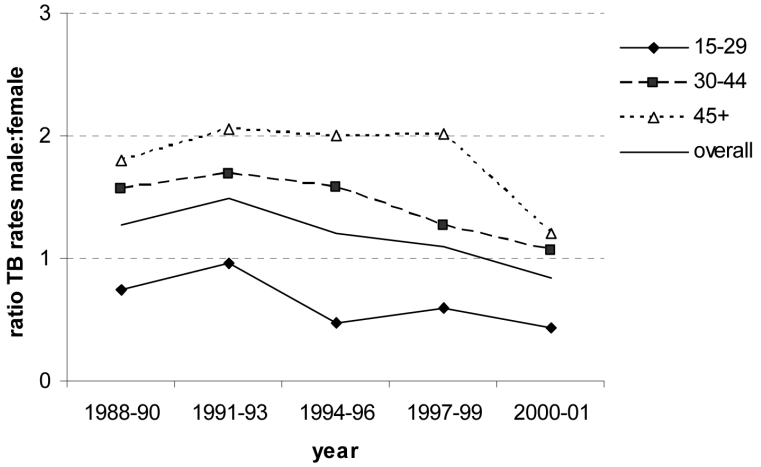

Figure 7.
Ratio of male to female cases of smear-positive pulmonary tuberculosis by age group over time.

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