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**Investigation of immune correlates, and impact of human
cytomegalovirus and immune activation, on tuberculosis
disease risk**

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Doctor of Philosophy

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DECLARATION OF OWN WORK

I, Lisa K Stockdale, confirm that the work presented in this thesis is my own.

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September 2018

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ABSTRACT

Background

Recent evidence implicates human cytomegalovirus (HCMV), immune activation and inflammation as risk factors for tuberculosis (TB) disease. Evidence also points towards the underappreciated importance of humoral immunity in protection against TB.

Methods

This PhD thesis uses stored serum samples from an established rural Ugandan cohort to investigate associations between HCMV, inflammation and TB. Firstly, HCMV epidemiology is investigated, and levels of mycobacterial antibodies are characterised in a cross-sectional study design including over 2,000 individuals. Secondly, a nested case-control study design was employed to explore longitudinal relationships prior to TB diagnosis in 343 matched samples.

Results

This work has characterised the HCMV seropositivity in this population and found that 95% of individuals are infected by age 5. Mycobacteria-specific antibody levels increased in all individuals until a plateau was reached at approximately age 20. HIV positivity and high HCMV IgG levels were independently associated with decreased levels of mycobacterial antibodies.

HCMV IgG levels, but not other chronic herpes infections EBV and HSV, are associated with increased risk of TB up to 14 years prior to TB diagnosis. HCMV IgG levels, but not EBV or HSV, are positively correlated with the inflammatory serum marker IP10.

Increased antibody avidity against the mycobacterial antigen mixture, PPD, showed a directional trend towards decreased risk of TB at early time points prior to TB diagnosis.

Conclusions

Data presented in this thesis confirm that HCMV burden is high in this rural Ugandan population. Anti-mycobacterial antibody levels increase with age, suggestive of cumulative exposure to either antigenically related non-tuberculous mycobacteria (NTM) or to *M.tb* itself. This body of work provides more evidence that HCMV infection is associated with TB disease risk. As opposed to a simple binary seropositive measurement, I have found that risk of TB disease is associated with magnitude of HCMV IgG response.

In summary, the data presented in this thesis:

1. Characterise HCMV infection in this large rural Ugandan cohort
2. Provide data on anti-mycobacterial antibody levels by age
3. Add to the evidence that HCMV infection is a risk factor for TB disease, and highlights the link between TB disease risk and magnitude of HCMV IgG level
4. Suggest that, in studies of TB risk, the wider immune environment (specifically inflammatory markers) should be investigated
5. Suggest that measures of antibody quality, not just quantity, should be investigated in the search for immune correlates of TB disease risk.

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GLOSSARY

Ag85A	Antigen 85A
Ag85B	Antigen 85B
APC	Antigen presenting cell
BCG	Bacille Calmette-Guérin vaccine
CFP10	10-kDa culture filtrate protein of <i>M.tb</i>
CFU	Colony-forming units
CRP	C-reactive protein
CVD	Cardiovascular disease
DC	Dendritic cell
EBV	Epstein Barr virus
ESAT6	6-kDa early secreted antigenic target of <i>M.tb</i>
HCMV	Human Cytomegalovirus
HIC	High-income country
HIV	Human Immunodeficiency virus
HSV	Herpes simplex virus
IFN- γ	Interferon gamma
Ig	Immunoglobulin
IGRA	Interferon- γ release assay
IL	Interleukin
LAM	Lipoarabinomannan
LMIC	Low- and middle-income country
LLOQ	Lower limit of quantitation
LTBI	Latent TB infection
M.tb	Mycobacterium tuberculosis
MHC	Major histocompatibility complex
MDR-TB	Multidrug resistant TB
MGIA	Mycobacterial Growth Inhibition Assay
MSMD	Mendelian susceptibility to mycobacterial infections
MVA	Modified Vaccinia Ankara
NHP	Non-human primate
NK	Natural killer cell
NTM	Non-tuberculous mycobacteria
OD	Optical density
PBMC	Peripheral blood mononucleocytes
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline with Tween
PPD	Purified protein derivative
RD1	Region of differentiation
SCID	Severe Combined Immunodeficiency
TB	Tuberculosis
TNF- α	Tumor necrosis factor alpha
TST	Tuberculin skin test
TTP	Time to positivity
WHO	World Health Organisation
XDR-TB	Extensively drug resistant TB

CHAPTER 1: INTRODUCTION

In this first chapter, I will introduce Tuberculosis disease and its etiological agent *Mycobacterium tuberculosis (M.tb)*, as well as provide an overview on current diagnostics, treatment and vaccines. A brief overview of the immune response to *M.tb* will separate the cell-mediated and humoral immune responses and detail immune correlates.

Next I will discuss Human Cytomegalovirus (HCMV) and provide an overview of its important role in immune activation and senescence before detailing the specific project aims and objectives which encompass these two pathogens.

1.1 Tuberculosis

1.1.1 *Mycobacterium tuberculosis* – the pathogen

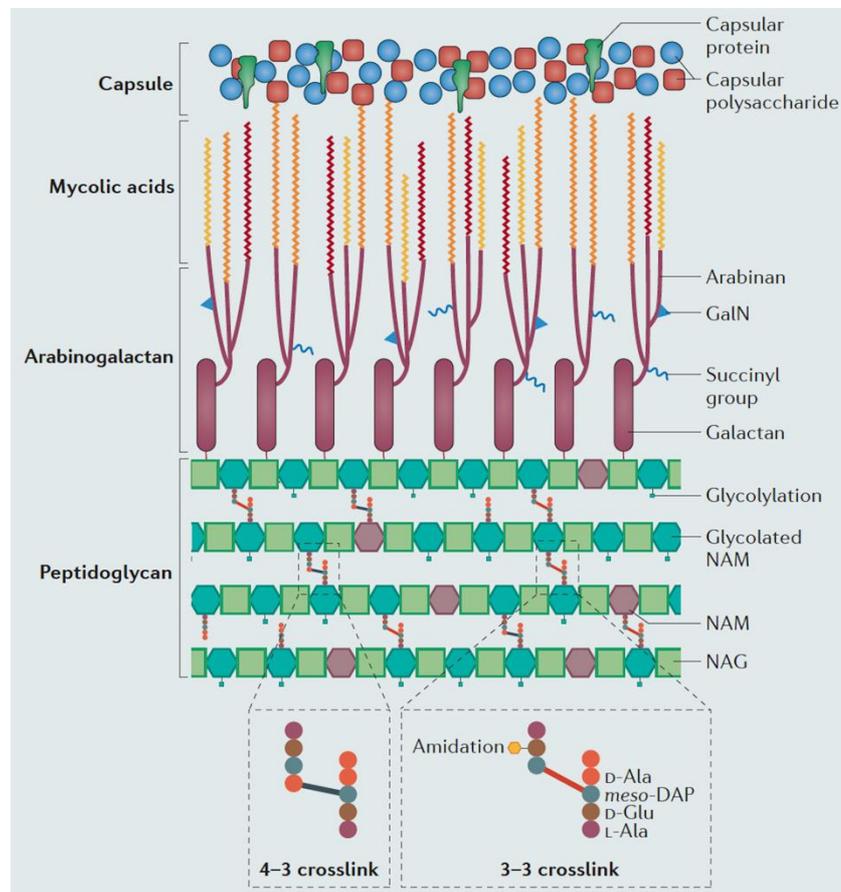
Tuberculosis (TB) in humans is caused by the bacteria *Mycobacterium tuberculosis (M.tb)*. First identified as a pathogen in 1882 by Robert Koch, *M.tb* is part of the acid-fast genus *Mycobacterium*, characterised by the mycolic acid-containing, nearly impermeable cell walls. *M.tb* is very slow growing (generation time of 20-24 hours [1]) and is able to remain dormant within an individual for decades before reactivating into active tuberculosis.

The genus *Mycobacterium* also comprises *M. leprae*, the etiological agent of leprosy, *M. bovis*, which causes disease in cattle, as well as approximately 120 atypical or Non-Tuberculous Mycobacteria (NTM), which are soil-dwelling saprophytes [2]. It has been found that around a third of these NTMs are capable of causing localized disease in the lungs, lymph glands, skin, wounds or bone, some can cause disseminated disease in immune-compromised individuals [3,4].

Tubercular deformities found in individuals with TB in the bone who died more than 4,000 years ago suggests that the disease was common in Europe and the Middle East. Recent genomic analyses indicate that TB probably emerged 70,000 years ago and accompanied human migration around the world [5].

The complete genome sequence of *M.tb* was published in 1998 and comprises around 4,000 genes, many of which are involved in production of enzymes involved in fatty acid metabolism [6]. This large genetic investment in lipid synthesis and breakdown has given rise to novel biosynthetic pathways to generate cell-wall components contributing to the highly hydrophobic cell envelope; the major constituents being peptidoglycan, arabinogalactan and mycolic acids [7] (Figure 1-1).

Figure 1-1 The mycobacterial cell wall. Taken from Kieser & Rubin, Nature Reviews Microbiology (2014) [8]



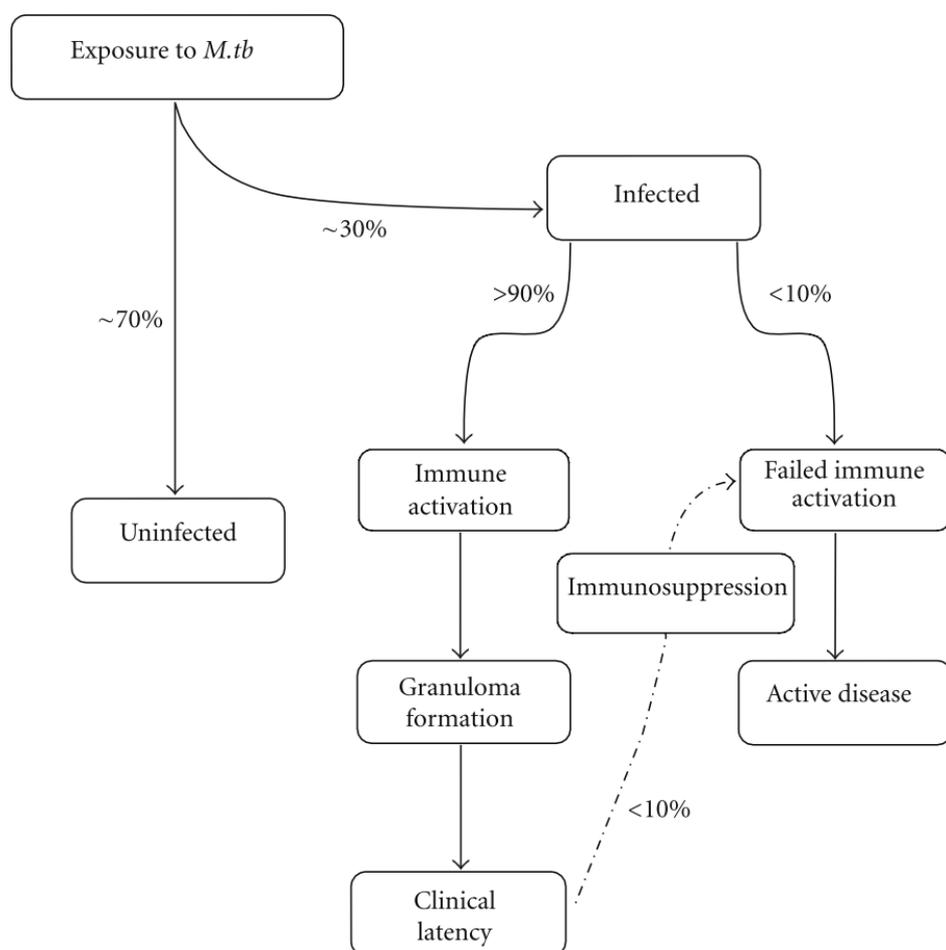
The lipid-rich cell wall acts as a barrier to drugs, making treatment difficult [9]. *M.tb* is able to withstand harsh intracellular conditions by virtue of the DosR regulon; a set of approximately 48 co-regulated genes induced by hypoxia which facilitate entry to a latent state characterised by very low metabolic turnover [10].

1.1.2 TB Disease

Upon exposure to *M.tb* bacilli, it is estimated that 70% of individuals remain uninfected (Figure 1-2). Of the 30% that do become infected, over 90% of individuals are able to contain the bacilli by forming a physical barrier known as the granuloma [11] which leads to latent

infection. While the majority of individuals exposed to *M.tb* do not develop TB disease, HIV and other forms of immunosuppression increase the likelihood of developing disease, increase the severity of symptoms, and decrease the time to progression [1,12].

Figure 1-2: TB disease progression. More than 90% of infected individuals enter a clinically asymptomatic latent state. Taken from Shaler *et al*, Clinical and Developmental Immunology (2012) [11]



TB is spread by symptomatic individuals with pulmonary TB when *M.tb* bacteria are expelled in droplets during coughing fits. TB can be pulmonary (affecting the lungs) or extra-pulmonary (affecting a range of other organs such as pleura, lymph nodes, skin, joints, bones or meninges;

causing TB meningitis). The World Health Organisation (WHO) estimates that extra-pulmonary TB represents 15% of all TB cases [13] however this is likely an underestimation given the difficulties in diagnosis [14].

Active TB disease may follow soon after initial infection, resulting in what is termed “primary” disease, or many years later due to reactivation of latent infection. For latently infected individuals, lifetime risk of developing clinical TB disease is estimated at 5-10% [15]; among those co-infected with HIV, this increases to a 10% annual risk [16].

The existence of a spectrum of TB disease has long been noted and is based on differences in clinical outcome, pathology of disease and immunological responses [17]. It is thought that a quarter of the world’s population is infected with TB and yet show no clinical symptoms [18]. This figure equates to 1.7 billion individuals who are latently infected with *M.tb* and are at risk of developing active disease, and thus transmitting TB to others.

Notifications of disease due to the approximately 120 NTM have increased since the early 1980’s, coinciding with the AIDS epidemic. Some NTM infections in HIV positive individuals are clinically indistinguishable from *M.tb*, and some cause a higher proportion of disseminated disease which is harder to diagnose [19]. In general, NTM are inherently resistant to standard TB chemotherapy.

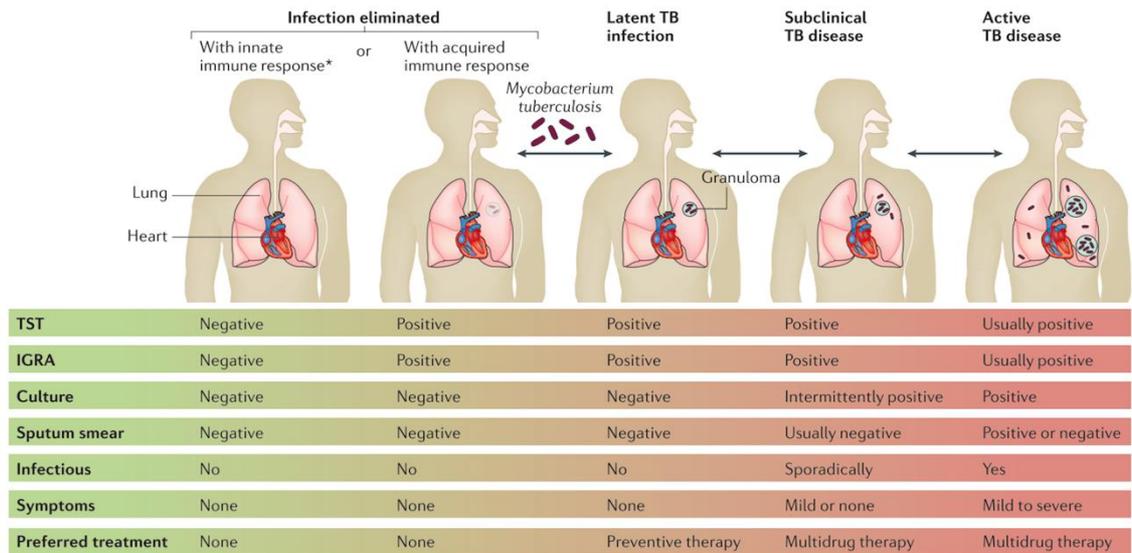
It is thought that exposure to NTMs may contribute to variability of effectiveness of the only licensed TB vaccine, BCG [20].

1.1.2.1 Latent TB Infection

Latent infection is thought to comprise a continuous spectrum from containment of *M.tb* bacilli with priming of a T-cell response, to clinical disease with symptom severity positively correlated with bacterial load (Figure 1-3) [21,22]. Targeting of preventative treatment to latently infected individuals most at risk of developing active TB disease would be an effective

way to stop transmission, however latent phenotypes are not readily elucidated by current diagnostic techniques [23].

Figure 1-3: Spectrum of TB infection ranges from sterilising immunity to high bacterial load and clinical disease. Taken from Pai *et al*, Nat Rev Dis Prim. 2, 1-23 (2016). [21]



1.2 TB epidemiology

TB is the leading cause of death from a single infectious agent, currently ranking above HIV/AIDS. In 2016, an estimated 10.4 million people were ill with active TB disease, of whom 6.3 million (61%) were newly diagnosed in that year. There were an estimated 1.34 million TB deaths in 2016; 85% of these occurred in the WHO African and South-East Asian regions (Figure 1-4) [13]. Without treatment, the 10-year case fatality rate for HIV negative sputum smear positive primary TB is 70% [24].

Figure 1-4: Estimated TB incidence for countries with at least 100,000 incident cases 2016.

Taken from WHO Global TB Report 2017 [13]

Estimated TB incidence in 2016, for countries with at least 100 000 incident cases



According to WHO, in 2016 90% of the 10.4 million reported cases were adults and 65% were male. Five countries accounted for 56% of the global total; India, Indonesia, China, the Philippines and Pakistan, with India, Indonesia and China having the largest number of incident cases [13]. Although TB is a notifiable disease, in areas with poor access to healthcare and no active case-finding, these numbers are likely an underestimate of the true burden.

With respect to age distribution, a large observational study which enrolled over 82,000 tuberculin skin test (TST) positive children in Puerto Rico between 1949-1951 and followed them for 8-20 years [25], found a distinct age distribution of disease which was confirmed in a systematic review of studies from the pre-chemotherapy era [26]: a peak in the first 1–4 years of life, followed by a trough until early puberty, rising to a second peak around the age of 20 years.

High incidence in the very young is important since risk of developing active TB disease increases with younger age at which initial infection occurs [25]. Diagnosis in this group is often problematic due to the low bacterial count found in TB infection in children [27] resulting in under-reporting and delays to treatment initiation.

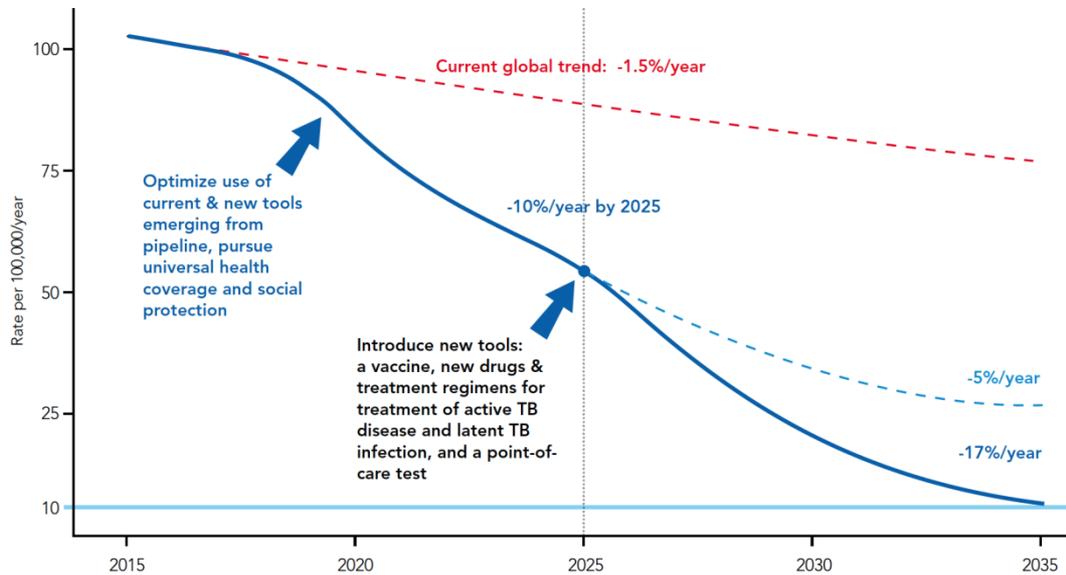
In many countries, TB case notification is twice as high for men as it is for women and this is not thought to be completely accounted for by differences in health seeking behaviour [28]. Indeed a recent TB household risk study in Germany revealed active TB was more prominent in men [29], and an animal study showed a preponderance of male mice in accelerated disease progression upon aerosol *M.tb* challenge [30].

1.2.1 End TB Strategy

In 2013, the WHO published a roadmap of how to meet the goal of ending the global TB epidemic [31]. The target was a 95% reduction of deaths due to TB by 2035. The three pillars of this strategy consist of integrated, patient-centred TB care and prevention, bold policies (including universal health coverage and social protection), and intensified research and innovation. Specifically, the document focusses on research to deliver better diagnostics, safer and more effective treatment for both active and latent TB and effective pre- and post-exposure vaccines.

Models suggest that in order to meet these goals, the TB field needs to optimise current tools but also it will require the introduction of a new vaccine, new drugs and treatment regimes, as well as a new point-of-care diagnostic (Fig 1-5).

Fig 1-5: Desired decline in global TB incidence rates to reach the 2035 targets (taken from End TB strategy) [32]



1.2.2 Diagnosis

Developed more than 100 years ago, the most common way to diagnose contagious TB is the cheap and simple sputum smear microscopy in which bacteria are visualised under a microscope from sputum samples. This is a relatively insensitive technique, firstly requiring that a sputum sample be produced (which is not straightforward in children), and necessitates at least 5,000 bacilli per millilitre of sputum (which is problematic in HIV-infected individuals due to reduced cavitation and transfer of bacilli into respiratory secretions). Sensitivity is reduced in patients with extra-pulmonary TB and this technique is further limited by its inability to distinguish between viable and non-viable TB, as well as between TB and NTM [33,34].

Although longer, more complex and expensive, culture methods allow for distinction between viable and non-viable bacteria, as well as between TB and NTM. Culture diagnosis can detect

cases prior to becoming infectious and hence often increases the number of cases identified by up to 50% compared with sputum smear microscopy [33]. Further culture in the presence of anti-TB drugs can determine drug sensitivity and resistance.

Genotypic methods such as the Xpert MTB/RIF are fast, high throughput and are useful in populations with a high incidence of HIV. The Xpert MTB/RIF is a cartridge based nucleic acid amplification assay which detects TB and Rifampicin resistance directly in sputum in 2 hours. Although more expensive than sputum smear and culture, the enhanced sensitivity of Xpert MTB/RIF contributes to its cost effectiveness, especially in HIV infected individuals [35].

The Tuberculin Skin Test (TST or Mantoux test) has been used for more than a century as an initial screening test for both latent and active TB. A cellular immune response to antigens present in an *M.tb* extract, tuberculin (collectively known as protein purified derivative - PPD), causes a hard induration at the point of subcutaneous injection. A positive result is indicative of an increased risk of developing TB, or currently having TB [36]. Despite its widespread use, false positives in BCG-vaccinated individuals are common [37].

Interferon Gamma Release assays (IGRA) such as QuantiFERON Gold and T-SPOT.TB are used to detect latent TB and function on the basis of *ex vivo* blood lymphocyte Interferon Gamma (IFN- γ) release after stimulation with PPD. Although unable to differentiate between active and latent TB, IGRAs have higher sensitivity than TST in BCG vaccinated populations [38] and do not rely on production of sputum.

An ideal diagnostic would be a cheap, fast and low-tech assay using an easily-accessible biological sample. Although still in development, a 'dipstick' assay measuring the *M.tb* cell wall

lipopolysaccharide, lipoarabinomannan (LAM), has promise as a new diagnostic in HIV positive individuals [39] and an alternative skin test using antigens ESAT6 and CFP10 has shown good diagnostic efficacy in a field-friendly format [40].

The lack of a specific and sensitive diagnostic to differentiate stages of active and latent infection has hampered the advancement of knowledge into TB epidemiology as well as drug and vaccine development.

1.2.3 Treatment

First-line therapeutics for TB include isoniazid, rifampicin, pyrazinamide, ethambutol and streptomycin. All are associated with hepatotoxicity and related side effects, and were developed in the early twentieth century. The World Health Organisation (WHO) recommends a 6 month regimen for treatment of drug sensitive TB: consisting of 2 months of isoniazid, rifampicin, pyrazinamide and ethambutol, followed by 4 months of isoniazid and rifampicin [41].

Injectable agents such as kanamycin, amikacin, capreomycin, along with the fluoroquinolones levofloxacin, moxifloxacin, ofloxacin, and other bacteriostatic drugs are termed “Second-line” therapeutics and are used in drug resistant cases of TB [42].

Multidrug resistant (MDR) TB is defined as resistance to isoniazid and rifampicin. Extensively drug-resistant (XDR)-TB is resistant to at least one fluoroquinolone and one injectable second-line anti-TB drug in addition to isoniazid and rifampicin. Of the 6.3 million new cases of TB in 2016, over half a million of those are thought to be MDR-TB strain, with up to 30% of new TB cases being MDR-TB in former Soviet Union hot-spots [13].

Recommendations for treatment of MDR-TB are not straightforward, and depend on the capacity of the country to carry out drug resistance testing, as well as the availability of second-line therapeutics, but the minimum treatment duration is eight months [43]. The estimated direct cost of treating MDR-TB is eight times, and XDR-TB 25-35 times higher than treating drug-sensitive TB [44]. The potentially devastating indirect costs to patients include travel to clinics and loss of productivity.

In summary, TB treatment regimens are long, expensive and cause side-effects which have ramifications on compliance. Development and roll-out of an efficacious vaccine would greatly aid the battle against drug resistance.

1.2.4 TB Vaccines

There exists only one licensed TB vaccine: Bacille Calmette-Guérin (BCG) is a live attenuated strain of bovine tuberculosis introduced in 1921 [45]. Although efficacious in protecting children and infants from disseminated TB and tuberculous meningitis, it has shown varied protection in adults against the most transmissible pulmonary form [20,46]. The large variation (0-80%) in protection elicited by BCG is thought to be multifactorial, with age at which BCG is given and previous exposure to mycobacteria (either *M.tb* or NTM, which has been linked to distance from the equator) being implicated [20].

A large trial of BCG involving approximately 50,000 people in the UK gave a protective efficacy against pulmonary disease risk ratio (RR) estimation of 0.22 (CI 95% 0.16-0.31) during the first five years of the study [47] (BCG vaccinated individuals were 78% less likely to be diagnosed with TB compared to unvaccinated individuals). The final 15 years of the 20 year study period however coincided with a general decrease in prevalence and incidence of TB in the UK and so

the predicted benefit of BCG was deemed to be decreased as well [48]. Conversely, the Chingleput study in India found an absence of any clinical benefit in over 200,000 individuals, with an RR of 1.05 (CI 95% 0.88-1.25) [49].

A recent systematic review of all published BCG efficacy trials found that BCG was protective against pulmonary TB. Greater protection was seen when BCG was given earlier in life, and when given to individuals with no TST evidence of prior exposure to mycobacteria [20]. Protection against meningeal and miliary TB was greater than pulmonary TB, and again, was greater when given to younger infants with lower risk of prior mycobacterial exposure. The protective effect against pulmonary TB was seen to change with latitude: protection was absent or low in trials conducted near the equator, and higher in those conducted more than 40° distant [20].

Proposed explanations for the association of variable BCG efficacy with latitude include host genetics, nutritional status and vitamin D availability, however it is now thought that the fact that NTMs are more prevalent closer to the equator may be the main driver behind the effect seen [50]. It is thought that exposure to NTM or *M.tb* itself, prior to BCG vaccination could lessen its efficacy [20].

Evidently there is a need to develop more efficacious vaccine candidates. Ideally, a vaccine would protect against both infection with *M.tb* in exposed persons and the development of disease in those who have already been infected. As of 2017 there were 12 vaccine candidates in clinical trials. The pipeline consists of products designed to replace BCG (prime vaccines), improve upon BCG (prime-boost vaccines), or shorten TB chemotherapy (immunotherapeutic vaccines). Some are being developed as prophylactic vaccines in very young infants prior to exposure to *M.tb* and some as therapeutic vaccines in previously exposed individuals [51].

There have been very few large-scale efficacy studies of developmental TB vaccines. Results of a Phase 2b trial with a fusion protein plus adjuvant (M72 AS01E) are expected in 2018 and the results of a pre-proof of concept prevention of infection study were published in July 2018 for the subunit vaccine H4:IC31 [52]. Another was a virally vectored prime-boost vaccine based on Modified Vaccinia Ankara (MVA) subunit vaccine, MVA85A [53]. The vaccine contains the gene for highly immunogenic Antigen 85A (Ag85A), a protein secreted by *M.tb*, as well as other mycobacteria, which is involved in maintenance of cell wall integrity through mycolic acid transfer. The vaccine was not found to confer any additional protection above BCG in infants (n=1399 MVA85A, n=1398 placebo). The lack of efficacy was not predicted by either mouse nor non-human-primate (NHP) animal studies [54] and, in the absence of a reliable correlate or biomarker of protection from, or risk of, disease, large and expensive clinical trials are the only way to test clinical efficacy (see section on 'Immune Correlates and Biomarkers' below).

BCG is the only vaccine thought to function primarily through induction of a T-cell response [55], whereas all licensed vaccines against other pathogens rely upon induction of antibodies to exert a protective effect [56]. The divergence of immunology from vaccinology in the infancy of both fields of research has led to the empiric development of effective vaccines with little understanding of the underlying immune mechanisms. Given the failure to develop successful vaccines against global diseases such as HIV and TB, there is increasing cooperation to dissect protective immunological pathways [57].

1.3 Immune response to TB

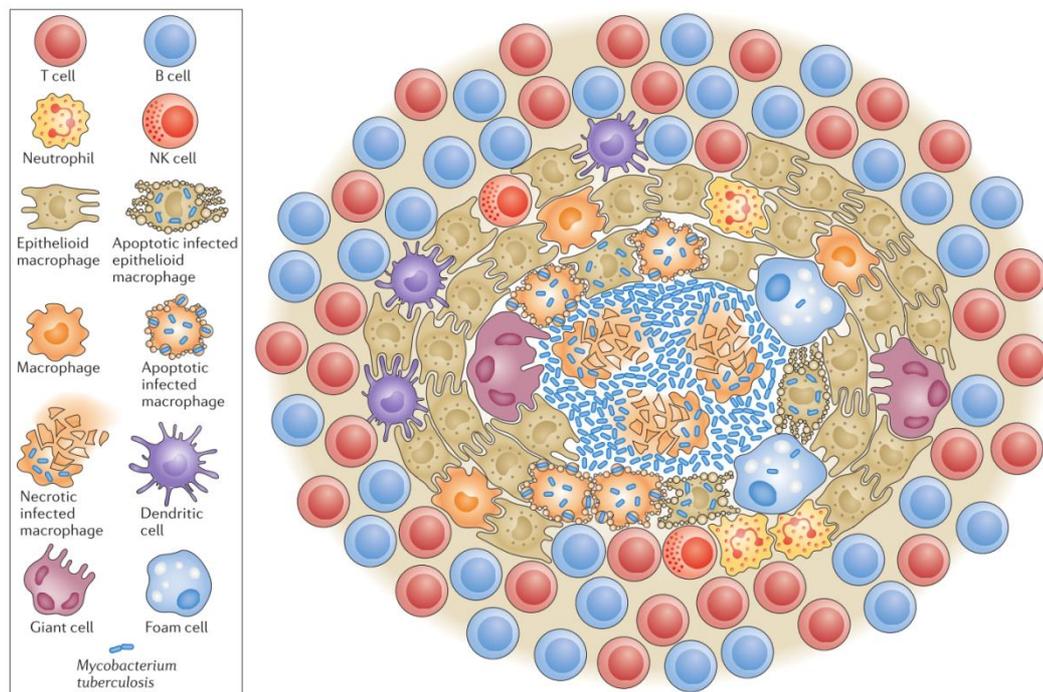
When *M.tb* bacilli are spread by aerosolised droplets and inhaled into the airways of a new host, the initial host response is characterised by an influx of phagocytic cells including resident alveolar macrophages, lung dendritic cells (DCs) and neutrophils [58]. *M.tb* bacilli are

taken up by a variety of cell types including DCs, macrophages, neutrophils, monocytes and epithelial type II pneumocytes [59]. Infected DCs migrate to the local draining lymph node 8-12 days after infection where they activate antigen-specific T-cells and drive differentiation towards an inflammatory Th1 phenotype [60].

M.tb has evolved a variety of immune evasion strategies including; induction of macrophage production of the immune regulatory cytokine IL-10 which prevents phagosome maturation and phagolysosomal fusion [60]; ESAT-6 directed sequestration of host beta-2-microglobulin resulting in the down-regulation of macrophage antigen presentation through MHC Class-I [61]; lipoarabinomannan (LAM) signalling through alveolar macrophage mannose receptor to reduce the cellular secretion of pro-inflammatory cytokines TNF- α and IL- β and chemokines MCP-1 and IP-10, thereby impairing recruitment of innate immune cells to the lungs [62,63], and delaying migration of antigen presenting cells (APC) to the draining lymph node, resulting in subsequent delays in T-cell priming [11].

The delayed onset of the adaptive immune response to *M.tb* allows the migration of infected cells into tissues and their aggregation into granulomas with accompanying bacterial proliferation, before activation of mycobacterial-specific T-cells [64,65]. The granuloma is a general term to describe an organized aggregate of mature macrophages that arises in response to a persistent stimulus. In TB, the granuloma consists of a dynamic array of cell types attempting to segregate intra- and extra-cellular bacteria from the host (Figure 1-6) [66].

Figure 1-6: Structure of the TB granuloma. Taken from Ramakrishnan, Nature Reviews Immunology, 2012 [66].



The large number of TB cases around the world is evidence that the sequestration of bacteria in these complex aggregates often fails. Observational studies in humans, and research in mice, evidence the importance of the inflammatory cytokine IFN- γ in granuloma formation and maintenance. IFN- γ -deficient mice are unable to develop granulomas [67] and humans with Mendelian susceptibility to mycobacterial infections (MSMD) were found to have variants in genes involved in IFN- γ signalling pathways [67].

In addition, the bacteria themselves have evolved ways of manipulating the ability of the host to produce IFN- γ : the early secreted proteins ESAT-6 and CFP-10, which form two of the gene products of a 9.5-kb section of *M.tb* DNA called Region of Deletion 1 (RD1), by virtue of the fact that they are deleted in BCG and NTM, are involved in virulence and pathogenesis of *M.tb* infection [68]. ESAT-6 contributes to virulence by inhibiting T-cell IFN- γ production [69].

While granulomas are a host defence mechanism to ‘wall off’ invading *M.tb*, the necessary inflammatory response needed to contain infection can also cause immunopathology in the form of necrosis. Infection with *M.tb* can elicit macrophage death in two ways: apoptosis whereby the cell retains an intact plasma membrane and bacteria are destroyed, or necrosis, where cell lysis allows bacteria to spread and infect other cells [60]. The type of cell death that results after initial infection is thought to depend on pathogen and host factors (the more virulent strains induce more necrotic cell death). Ultimately, TB morbidity and mortality is thought to result from an aberrant and excessive host immune response that is both pathologic in consequence yet ineffective in containing the pathogen [70].

Being a disease associated with high human morbidity and mortality, and with poor treatment options, TB human challenge models are considered unethical. Much of our knowledge of the immunology of tuberculosis infection has come from observational studies in humans and experimental animal studies, with the most important species being the mouse. While the mouse model has been crucial in demonstrating the protective role of CD4⁺ T-cells, IFN- γ , and TNF- α , there are limits to the use of genetically inbred laboratory mouse studies to any human pathology, and the same is seen in TB [71]. The currently used mouse strains are particularly poor models for *M.tb* lung granuloma formation and so many groups prefer to use guinea pig or non-human primate (NHP) alternatives.

1.3.1 Cell mediated responses

T-cell immunity is critical for protection against TB. The HIV epidemic in humans has highlighted the crucial role of CD4 T-cells: the preferential depletion of *M.tb*-specific CD4⁺ T cells is thought to contribute to the susceptibility of HIV positive people to TB disease [72].

Indeed, an increased 1.43 (95% CI 1.16–1.88) fold risk of TB incidence has been associated with every 100 cells per mm³ decrease in CD4 cell count [73]. In a TB challenge mouse model, CD4+ T-cell, or MHC-II deficient mice, were unable to control mycobacterial growth. CD8+ T-cells are also thought to be important in protection against TB disease through the production of IFN- γ which in turn plays a role in the activation of macrophages. However CD8+ T-cells cannot compensate for the lack of CD4+ T cells in a CD4+ deficient model [74].

IFN- γ is a mediator of macrophage activation and resistance to intracellular pathogens. As previously mentioned, its importance in TB was highlighted by recognition of mutations in IFN- γ receptors of highly susceptible individuals, as well as the presence of IFN- γ specific auto-antibodies acting to block induction of IFN- γ inducible genes [75,76]. The important role of Tumour Necrosis Factor α (TNF- α) in human TB control was illustrated in 2001 when 70 reports of reactivation of active TB in subjects with latent TB were filed after receiving the anti-TNF monoclonal antibody, Infliximab, for rheumatoid arthritis or Crohn's disease [77]. *TNF*^{-/-} knockout and mice depleted of TNF by treatment with anti-TNF antibodies resulted in fatal TB infection in previously resistant strains [78,79].

Although human observational studies and mouse models have shown that Th1 responses such as CD4+ T cells, IFN- γ and TNF- α are all important in progression of *M.tb* infection [80–82], these factors do not explain the large heterogeneity in clinical outcomes. More likely is that disease outcome is dependent on many host, pathogen and environmental interactions [60].

1.3.2 Humoral responses

In the late nineteenth century, early immunological theory separated out defence against intracellular, and extracellular pathogens, and so ensued the concept that they are moderated by cell-mediated, and humoral (comprising B-cells and antibody) immune responses respectively [83,84]. With the overwhelming evidence for the importance of cell-mediated immunity in TB, and the inconsistent results of passive serum transfer in the late nineteenth century [85], the role of humoral immunity in TB has been largely overlooked despite evidence of its critical role in many other intracellular pathogens (reviewed in [86]).

Data showing a lack of association between frequency and amplitude of classical Th1 cytokine responses [87], and release of IFN- γ [53] with protection against TB disease in South African infants following BCG have cast doubt over the theory that a cell-mediated response is the only important factor in protective responses against *M.tb* [88].

For the purposes of this introduction, B-cells and their soluble secreted product, antibody, will be considered separately in the next section.

1.3.2.1 B-cells

B-cell activation is necessary for their maturation into plasmablasts, which produce high affinity pathogen-specific antibodies, as well as maturation into memory B cells which are crucial for immunologic memory. Whereas T-cells can only recognise processed (normally protein) antigens, as a professional APC, B-cells are able to interact with antigen in the native form [89] which may be particularly important in TB considering the high lipid component in *M.tb* cell wall. Their interaction with CD4 T-cells in peripheral lymphoid tissue structures, called germinal centres, activate antigen-specific T-cells and, under the right conditions, the T-cells provide activatory signals to the B-cells [90]. Reports suggesting modulatory roles for B-

cells in TB [91,92], along with evidence that B cells themselves are a source of IFN- γ [93] thus promoting a Th1 response, have renewed interest in investigation of B-cells in TB [94].

This renewed interest in B-cells in TB coincided with the discovery of B-cell aggregates within the granuloma, which were reminiscent of germinal centre follicle-like structures, in the lungs of TB patients [95]. The finding that T-cells could be found in these B-cell clusters [96] and that the aggregates contained germinal centre-type markers [97] gave rise to the hypothesis that this could be a site of antigen presentation and B-cell maturation similar to a germinal centre [98]. Further evidence of the potential importance of B-cells came from; exacerbated lung pathology and disruption of granuloma architecture in B-cell deficient mice [97], the detection of both activated B-cells and mycobacteria-specific antibody-producing plasmablasts within the granuloma of TB-infected NHPs [99], and that humans with active pulmonary TB disease have lower circulating B-cell counts than uninfected individuals [100].

1.3.2.2 Antibodies

Investigations conducted in the late 1800's using sera from various animals to induce protection in different animals (sometimes of different species), unsurprisingly showed highly variable results [85]. However, despite our improved understanding that the *M.tb* bacillus is not limited to an intracellular niche [66], and so therefore accessible to humoral responses, along with the discovery that antibody mode of action is not limited to extra-cellular locations [101], the TB field has been slow to investigate the role of antibodies in disease progression.

Antibodies have mainly been investigated for their use in TB diagnosis. An accurate serological test that could provide rapid diagnosis of TB and in a suitable format (e.g. point-of-care) would be particularly useful both as a replacement for laboratory-based tests and for extending TB

diagnosis to lower levels of health services, especially those without on-site laboratories. Unfortunately, almost all the antibody-based assays are limited by sensitivity, especially in smear-negative TB patients (reviewed in [102,103]). Based upon a systematic review of the diagnostic accuracy of commercial tests for 67 pulmonary TB and 25 extrapulmonary TB studies [104], in 2011, the WHO “strongly recommended that these tests not be used for the diagnosis of pulmonary and extra-pulmonary TB” [105].

While it has been repeatedly observed that although single-antigen-based assays never achieve satisfactory sensitivity and specificity, more recently, measurement of a combination of antibodies has been investigated for potentially better assay performance with equally disappointing results [106]. Antibody responses to a panel of 10 extracellular and immunodominant *M.tb* proteins failed to show a consistent pattern of antigen reactivity among confirmed TB cases [107], and more recently, a high throughput multiplex assay containing 119 *M.tb* antigens, found no predictive value based on differences between IgG responses from children with active TB and ‘unlikely’ TB [108].

While antibodies may not have been the diagnostic panacea people had hoped, it is important to note the heterogeneity of diagnostic methodologies to which antibody-based assays have been compared. The methodology used to diagnose TB in many studies has changed over time depending on technological advances. Indeed, studies use an array of measures to distinguish various groups: occupational exposure [109,110], sputum smear microscopy [111], culture positivity [112,113], persistent cough [114], x-ray [107], response to anti-TB treatment [115], TST reactivity (also referred to as PPD skin test) [111,116], IGRA using cell-mediated responses to ESAT-6 and CFP-10 antigens [116], some studies do not detail method of diagnosis, only using “WHO TB diagnosis” group which comprises Confirmed, Probable or Suspected [117]. Along with the heterogeneity of TB diagnostic methods used, many studies are not

comparable to one another due to the use of different antigen mixtures from different strains of *Mycobacteria*, and at different stages of growth (see Table 1-1)

Table 1-1 – Examples of the array of *Mycobacterial* antigens used to study antibody responses in human TB cases

MIXTURES (different methods for extraction, different strains of <i>M.tb</i> , at different stages of growth [118])	INDIVIDUAL ANTIGENS
PPD [119–121]	38kDa [107,115,122] called TB72 in [109]
CFP [123,124]	Alanine dehydrogenase [115]
TBGL [125]	Rv2626c [115]
Mtb whole cell lysate [123]	16kDa [115]
Ag85 complex [123]	Ferredoxin A [115]
Cell-free protein extract [122]	ESAT6 [107,115,116]
NTM Sonicates [112,126]	14kDa [107] called TB68 in [109]
Mtb sonicate [117,124]	MPT63 [107]
BCG extract [121]	19kDa [107] called TB23 in [109]
H37Rv extract/sonicate [112,121]	MPT64 [107]
H37Ra sonicate [112]	MPT51 [107]
Precipitated mucopolysaccharides of NTM [112]	MTC28 [107]
	Ag85B [107]
	KatG [107]
	CFP10 [116]
	LAM [109,117,122,124,126,127]
	65kDa HSP [126]
	70kDa HSP [126]
	32kDa (also known as Ag85A) [113]
	DAT [111]
	PGL-Tb1 [111]

(NTM - non-tuberculous mycobacteria, HSP – Heat Shock Protein, DAT - diacyl-trehalose, CFP – Culture Filtrate Protein, TBGL – anti-tuberculous glycolipid antigen, MTC – *M.tb* complex, LAM – lipoarabinomannan, PGL – phenolglycolipid antigen)

Human antibody reactivity to *M.tb* is highly variable; comprehensive systems biology investigation of the *M.tb* proteome showed that sera from suspected TB cases recognized at least one of a panel of 484 proteins (representing only 10% of the total *M.tb* proteome) regardless of TB disease state [114]. Active TB was associated with reactivity to only 13 proteins, all of which are extracellular, and the authors concluded that human antibody reactivity to *M.tb* antigens is likely determined by the metabolic state of the pathogen [114]. Many who work in the TB field are skeptical of the role of antibodies in TB because of the lack of a clear phenotype associated with magnitude of antibody response, however epidemiologic studies have shown a decrease in anti-LAM antibodies during reported peak TB incidence of disseminated TB in children [117], as well as in HIV positive TB patients [128] suggesting a possible protective role for anti-LAM antibodies. Antibody against the secreted 38-kDa antigen best correlated with advanced, multibacillary disease, while the antibody against the cell-associated 16-kDa antigen (α -crystallin) is detected preferentially in asymptomatic, infected individuals [109,115,129,130].

More recently, mechanistic studies have uncovered a more nuanced role for antibodies; enhanced phagocytosis of mycobacteria in the presence of post BCG-vaccination serum [131] and the observation that, despite overlapping antibody titres against *M.tb* antigens in uninfected, latently infected and individuals with active TB disease, active TB patients had lower avidity antibodies against surface antigens compared to uninfected controls [132]. It has been shown that pre-treatment of virulent *M.tb* bacilli with immune sera prior to infection inhibits the blockade of phagolysosomal fusion which, during normal TB infection allows the bacilli to evade host killing [133]. In immunotherapeutic vaccine studies, it has been shown that passive transfer of serum from vaccinated mice to Severe Combined Immunodeficiency (SCID) mice resulted in significant reduction of lung colony forming units (CFU) and reduced abscess formation. Authors concluded that antibodies might be used in conjunction with drug

therapy to treat tuberculosis [134,135]. A study carried out in Mexican Indians found that positive Ag85A antibody responses prior to initiation of anti-TB treatment was associated with less advanced disease and with subsequent responsiveness to chemotherapy in TB patients [136]. Following a developmental TB vaccine (MVA85A) which was not found to confer additional protection above BCG in a Phase 2b infant efficacy trial [53], a secondary study was initiated which aimed to identify correlates of protection based on pre- and post-vaccination stored cells and sera for cases and controls. Exploratory analysis suggested that Ag85A-specific antibodies were associated with reduced TB disease risk (estimated odds ratio 0.62 per 1 log₁₀ unit increase, $p = 0.019$) up to three years prior to diagnosis [137]. The majority of studies measure an immunoglobulin (Ig) G response however IgA may also be able to discriminate between *M.tb* infected and non-infected subjects [138–140]. A study investigating the qualitative differences in mycobacteria-specific antibodies in active TB patients found a difference in the sugar residues on immunoglobulin molecules [141]. These studies point towards the need for measurement of responses against standardised antigens and, in addition to measures of antibody quantity, investigate other aspects of antibody quality.

In summary, early studies investigating the role of antibodies in TB were hampered by lack of standardisation of methods and reagents. More recent studies have moved on from measuring amount of antibody to attempting to measure functionality of antibody. Results from these studies show that while antibodies may not be sufficient for protection against TB, they may well be necessary, and definitely warrant further research.

Next, I will discuss the need for accurate read-outs of disease progression, treatment efficacy and vaccine-induced protection. I will then introduce the current methods used to investigate these correlates or biomarkers.

1.3.3 Immune Correlates and Biomarkers

In order to achieve the ambitious goals set by the 'End TB Strategy' [32], there is a critical need to identify early changes indicative of risk progression to, or protection from, active TB disease. These changes, termed correlates or biomarkers, should be measurable in an easily accessible biologic sample and would direct treatment and provide read-outs from vaccine development studies. In many cases, multiple biomarkers are needed to identify a meaningful "biosignature". Given the difficulties in interpretation of animal models in TB research, it will be important to use such biosignatures as surrogate markers for the protective efficacy of vaccines, as correlates of protection.

A common immunological parameter measured in TB vaccine programmes is the cytokine profile of T-cells, knowing that a Th1 response is important to risk of disease - specifically IFN- γ release from T-cells stimulated *ex-vivo* with mycobacterial antigens. However caution is needed when interpreting these results as a 2010 study of 29 unprotected and two groups of 55 protected infants which measured mycobacteria-specific T cell frequency and cytokine profile, found no correlation of IFN- γ , TNF- α , IL-2, and IL-17 production from CD4+, 8+ or $\gamma\delta$ T-cells [87]. Tighter TB disease definitions in a more recent study did however find that BCG specific IFN- γ release was associated with lower risk of TB disease [142]. The immune components already identified as playing a role in protection or risk of disease might not be the only useful correlates of protection against disease.

An ideal TB biosignature should be reproducible in a diverse patient population, should differentiate between active and latent TB, it should return to normal levels during successful treatment, and predict vaccine efficacy thereby providing endpoints for clinical trials [143]. Factors likely to affect biomarkers include patient factors (age, ethnicity, environmental

exposure to NTM, co-infections and immune status), pathogen factors (infecting strain, route of infection, site of disease and stage of disease), and sample factors (type of sample taken, sampling handling and time between collection and analysis) [144].

1.3.3.1 Gene Expression Signatures

Technological advances in methodologies such as gene expression analysis have allowed for measurement of global gene expression in TB cases. An analysis of eight global gene expression studies [145–152] was conducted comparing whole blood or PBMC signatures from individuals with active TB over time during treatment and with healthy infected controls, uninfected controls, as well as other inflammatory diseases [144]. In this study, the authors note the limitation of peripheral blood sampling and the likely differences between peripheral blood and site of disease. Nevertheless, the prominent activated myeloid cell signature seen in the circulation is noted to be probably due to the magnitude of the inflammatory response at site of infection [144]. The 409 genes identified as being associated with TB disease were overwhelmingly associated with patterns of inflammation, interferon signalling pathways and myeloid lineage cell activation, however these patterns were also found in other, non-TB, inflammatory conditions. Upregulation of T- and B-cell receptor signalling, as well as antibody Fc receptor signalling, again implicates humoral immunity in TB disease. In 2016, a large prospective cohort study identified a 16-gene signature of risk of TB progression in South African progressors (n=46) and matched controls (n=107), which was validated in separate South African and Gambian cohort [153]. This whole blood RNA signature may be useful in identifying individuals at risk and targeting interventions to prevent active TB disease.

1.3.3.2 Mycobacterial Growth Inhibition

An alternative to gene expression signatures is to measure an individual's ability to inhibit growth of *Mycobacteria* *ex vivo* in an assay known as the Mycobacterial Growth Inhibition Assay (MGIA) [154,155]. These assays measure the summative ability of all cell types in either whole blood or peripheral blood mononucleocytes (PBMCs) to control growth and so the exact immune mechanism does not need to be identified. This assay has been used to compare growth of mycobacteria following BCG [156] and MVA85A [157] vaccination in humans. Although the assay requires optimisation, it has promise.

1.3.3.3 Other Biomarkers

In addition to their investigation as diagnostics, mycobacteria-specific antibodies have been investigated as biomarkers for treatment success. As opposed to the simplistic measurement of antibody level only, a study in UK adults measuring fluctuations during TB treatment found an initial increase in antibody levels with a subsequent decrease, a broadening of epitope recognition, and found no changes in antibody affinity in successfully treated individuals [158]. Differences were seen between patients with drug resistant *M.tb* and individuals who went on to relapse. A study in Ugandan active TB patients and uninfected or latently infected controls found differing ratios of B-cell phenotype. Healthy controls had higher plasmablasts compared to memory B-cells whereas active TB patients had higher memory B-cells compared to plasmablasts [159]. Unfortunately this study did not include patients with other inflammatory conditions which may have caused the shift in B-cell phenotype irrespective of TB status, especially since the same group previously found measurable levels of mycobacteria-specific memory B-cells in uninfected individuals both with and without BCG vaccination [160].

In 2016, a correlates of protection study which followed infants for up to three years before development of active TB disease (n=50) and matched controls who did not develop disease

(n=136), found risk to be associated with increased frequency of CD4 T-cells with an activated phenotype, and reduced risk with frequency of BCG-specific IFN- γ -secreting T-cells and IgG against Ag85A [142].

In summary, research into immune correlates is progressing with some important findings in recent years. It is increasingly unlikely that a single biomarker will be identified as a correlate of risk of disease progression or a marker of vaccine-induced protection however the combination of many markers into a biosignature may prove to be a useful tool.

1.3.3.4 Immune Activation and TB

As previously discussed, a major finding from a 2016 TB immune correlates study was that risk of TB disease was associated with pre-vaccination (D0) increased frequency of activated CD4+ T-cells (OR=1.12, 95% CI=1.04–1.2, P=0.002) and CD8+ T-cells (OR 1.02, 95% CI=1.00-1.04, P=0.056), n= 50 (cases), n= 136 (controls) measured up to three years prior to development of active TB disease in BCG vaccinated South African infants. This finding of increased risk of active TB disease with an activated immune phenotype was independently verified in *M.tb* infected adolescents (OR=1.387, 95% CI=1.068–1.801, P=0.014, n=30 (cases) n= 59 (controls) [142]. In this study the authors also included measurement of Human Cytomegalovirus (HCMV) infection (measured by T-cell IGRA) and found that magnitude of T-cell response to HCMV was correlated with T cell activation.

As will be discussed in the following section, HCMV is a known driver of significant immune activation [161–163], immunosenescence [164–166] and associated with increased overall mortality [167] with outcomes seemingly having a dose-response relationship with magnitude of HCMV antibody level [168].

There is increasing evidence of a detrimental role of general immune activation on health outcomes. This has been investigated extensively in HIV, with immune activation being associated with increased overall mortality, cardiovascular disease (CVD), neurocognitive impairment, osteoporosis and frailty [169–175]. It has been found that immune activation is more predictive of survival than plasma virus burden [176], with effects of HIV-induced immune activation evident despite antiretroviral (ARV) treatment [177]. The harmful effects of immune activation appear to be compounded when an individual is infected with both HCMV and HIV, with the compound effect seen even due to HIV exposure and in the absence of actual infection [178]. Immune activation has also been investigated in the context of live attenuated yellow fever vaccine where vaccinated individuals from Switzerland and Uganda were compared [179]. Baseline levels of pro-inflammatory monocytes and exhausted NK cells were higher in the Ugandan volunteers and were associated with impaired cellular and humoral responses to vaccination [179]. Both latent and active TB are themselves a source of immune activation and in areas of high TB/HIV co-endemicity, latent TB-associated T-cell activation may contribute to HIV disease progression and exacerbate the HIV epidemic [180–182]. Levels of serum activation markers have been linked to risk of TB drug toxicity in HIV/TB co-infected individuals [183].

The excess risk of active TB disease in HIV infected individuals is well known, however the relationship between HCMV and TB is very much less well researched. This PhD thesis will investigate the association of HCMV infection with markers of inflammation, and try to determine the impact of these on TB risk.

1.4 Human Cytomegalovirus

Human Cytomegalovirus (HCMV), also known as human herpesvirus-5 (HHV-5), is a member of the *β-herpesviridae* subfamily which is widely distributed in human populations. HCMV transmission occurs through person-to-person contact. It can be transmitted transplacentally to neonates or through breast milk of an infected and shedding mother, by intimate contact and by transplantation from (or sharing syringes with) an infected individual [184]. It has been shown that young children shed HCMV virus in saliva and urine at high levels which may add to transmission between infants and adult care-givers [185]. Congenital HCMV infection is the leading cause of permanent hearing and neurological impairment as well as vision loss in infants worldwide [186]. Maternal primary infection or reactivation, especially during the first trimester, is particularly associated with adverse neonatal outcomes [187]. The incidence of congenital HCMV infection is estimated at between 0.7 and 5% of all births in low and middle income countries (LMICs) [188].

In LMICs, such as Uganda, prevalence of HCMV infection is high, with 95% of individuals infected by age five [189]. In the United Kingdom, 15% of 1-4 year olds, 30% of 20-29 year olds, and approximately 80% of the population is infected by age 65 [190]. Much of the research on the clinical importance of HCMV has been conducted in HICs, where HIV-associated HCMV retinitis [191], congenital HCMV disease [192] and transplantation [193] are all important manifestations of disease. Despite generally higher seroprevalence, less is known about the clinical importance of HCMV in LMICs. It is a common cause of pneumonia and meningitis in immune-suppressed hospitalised patients, and autopsy studies in Africa frequently find HCMV in the absence of overt HCMV disease [194]. An anticipated decrease in HCMV-associated clinical issues with increased roll-out of antiretroviral drugs in sub-Saharan Africa has disappointingly not materialized [195]. Detection of HCMV in blood correlates with progression to other AIDS-defining illnesses and death, even in the era of ART [196], and there

may be a benefit to empiric systemic treatment with anti-HCMV drugs such as ganciclovir [191].

In immunocompetent adults, HCMV infection rarely causes disease; however, once infected, the virus remains latent in a wide range of cell types including lymphocytes, myeloid lineage cells as well as smooth muscle cells and endothelial cells which line blood vessels [197]. Monocytes are a key host cell of HCMV during latency [198,199]. While monocytes are not permissive to viral replication, HCMV infection can drive cell differentiation to macrophages [200] which do support the full lifecycle of HCMV.

HCMV/HIV co-infection is common and HCMV infection is an important cause of severe non-AIDS events, including death, in HIV-infected individuals [201,202]. Epidemiological studies in high income countries (HICs) have found associations between HCMV infection and increased risk of mortality in older people [168,203]. Further studies have implicated chronic HCMV Infection as a risk factor for cardiovascular disease (CVD); a recent meta-analysis of studies conducted in HICs, estimated a 22% increased relative risk of CVD with exposure to HCMV [204]. In a UK setting, HCMV infection was associated with the development of arteriosclerosis [205] and an increase in blood pressure among older individuals [206].

In this thesis, the use of HCMV IgG measurement as a proxy for HCMV virus exposure is an assumption [202,207,208]. Evidence does, however, exist to suggest that this is not the case and viral shedding is not associated with antibody levels [209]. It is well known that HCMV infection, and shedding is associated with lower socioeconomic status [210–212] however, it has also been postulated that genetic predisposition, or other factors such as stress, could

induce an excessive inflammatory profile which may be associated with increased production of HCMV-specific antibody [210].

1.4.1 Impact of HCMV on the immune system

The immune response to HCMV infection is one of the largest ever documented [162,213]. As already mentioned, HCMV infection is linked to long-term health outcomes. These include CVD [167,206], cancers [214,215] and a range of cognitive disorders such as Alzheimer's [216,217]. A study of 105 twin pairs which measured over 200 cellular and serum characteristics found that most of the differences in immune parameters were due to non-heritable factors. The authors found that discordant HCMV infection status in monozygotic twins was found to be associated with differences in 58% of all parameters tested [207].

Latency in HCMV is not a silent process. Accumulating evidence suggests that the virus itself directs significant activity towards the maintenance of this state, and the expression of viral proteins occurs during non-replicative latency [218–220]. Some of these proteins are involved in manipulation of cellular immune recognition machinery, while others alter the cellular environment, contributing to an immune-suppressive environment (reviewed in [221]). Maintenance of HCMV in a latent state appears to be a very resource-intensive activity for the host immune system. Characterisation of the HCMV-specific T-cell population revealed that up to 30% of circulating CD4 and CD8 memory compartments are dedicated to the restraint of viral replication in HCMV seropositive individuals [222]. This phenomenon, termed 'memory inflation' depletes the available pool of naïve T-cells by pulling them into differentiated HCMV-specific memory phenotypes [165]. HCMV-associated activation of T-cells is linked to permanent high expression of IFN- γ and other Th1 cytokines to induce a chronic pro-inflammatory state associated with immune activation and senescence [213] and has been found to correlate to acute-phase response proteins such as CRP [162,223]. HCMV infection

has also been linked to the differentiation of Natural Killer (NK) cells [224,225], (an important cell type in early responses to infections, reviewed in [226]) impairing their ability to respond to cytokine stimulation in post-vaccination responses [227,228].

The relationship between HCMV and vaccine responses is not straightforward. Influenza vaccine is given to at risk groups and the elderly in many countries. HCMV has been investigated as a possible reason for the variable protection seen. One US study showed up-regulation of immune responses to influenza vaccine in HCMV positive younger people but down-regulation in HCMV positive older people [229], another showed higher anti-influenza serum responses in HCMV negative younger individuals and no difference in older people [230]. A study in the Netherlands found HCMV to have no impact on the poor responsiveness seen in elderly recipients of influenza vaccine [231] and in Poland, non-responsiveness was linked to a HCMV-induced pro-inflammatory environment [232]. It is interesting to note that the above studies use HCMV serostatus as a measure of infection status. It has been postulated that extent of exposure (reactivation and re-infection) to HCMV, as measured by HCMV-specific IgG titre, may be a better way to gauge correlation with HCMV infection [168,233]. Indeed, mouse studies have indicated a dose response to the initial inoculum, with compromised ability to clear heterologous viral infections with high initial exposure [234,235].

HCMV utilises its 225 genes to manipulate the immune system in many ways [236]. Initially, HCMV evades host immune responses via phosphorylation of the major immediate-early gene product UL122, thereby preventing its proteasomal degradation and presentation on the surface of infected host cells [237]. Other virally encoded proteins essential in HCMV immune evasion include; US6 which blocks peptide presentation, reducing CD8 T-cell recognition [238,239]; US2 which causes degradation of HLA-DR- α and DM- α , two essential proteins in the MHC class II antigen presentation pathway, thereby blocking CD4 T-cell presentation of viral

antigens [240]; UL111A; a homologue to the Th2 cytokine IL-10 which restricts differentiation of myeloid progenitors into dendritic cells [241]; and UL148 which inhibits CD58, an adhesion molecule critical in co-stimulation of effector cells [242] Notorious for down-modulating MHC Class-I, all herpes viruses, but HCMV specifically, rely upon Natural Killer (NK) cells (which lack the normal complement of MHC-I display) in the absence of cytotoxic T lymphocytic CD8 responses. This is evidenced by individuals with NK cell deficiencies [243]. HCMV infection impacts the maturation and differentiation of NK cells [244] however Investigation into HCMV interaction with NK cells is not well characterised. It is known that HCMV dedicates many genes to facilitate NK cell evasion [245], and an evolutionary expansion of HCMV-encoded NKG2D ligands (molecules which regulate NK cell function) likely conferred a genetic advantage [246,247].

1.4.1.1 HCMV and TB

The impact of HCMV infection on the immune-system is far-reaching and there is evidence to suggest that immune control mechanisms important in defence against *M.tb* are subverted in HCMV infection. Epidemiological studies have shown a possible link between HCMV and TB, however these studies investigated the impact of TB upon HCMV antibody levels, not the impact of HCMV upon TB; a study in Nigeria found significantly more HCMV seropositive people among TB patients compared to non-TB hospitalised patients, with those TB patients having higher titres of anti-HCMV antibodies [248]. Elevated levels of HCMV antibodies were found in plasma from patients with pulmonary NTM disease [249]. A study in Russian children and adolescents found that pulmonary TB patients were three times more likely to be HCMV infected compared to non-TB controls [250]. A strong cellular response against either HCMV or Epstein Barr virus (EBV) predicted a favourable anti-TB therapy in hospitalised TB patients in Tanzania [251] however these HCMV responses were likely an indicator of general immune fitness and therefore may have been an independent predictor of survival.

1.5 Aims and Research Objectives

There remain many gaps in our knowledge as to the reasons for some individuals' ability to control infection with *M.tb* while others develop active disease. Given recent evidence, there has been renewed interest in the role of humoral immunity, specifically mycobacteria-specific antibodies, in TB disease. The evidence implicating immune activation in predicting onset of TB disease has also sparked interest from the TB community as a possible component of a biosignature of risk. The knowledge that HIV is a major risk factor for TB and that both diseases induce a co-aggravating environment of immune activation and cellular differentiation points towards the potential importance of other co-infections acting through a similar route. Not only has HCMV been shown to be a major driver of immune differentiation and activation, the route by which HCMV achieves this appear to be independent of, and in addition to, that seen in HIV [178].

The central hypothesis of this thesis is that infection with HCMV will be associated with risk of TB disease and affect a putative protective antibody immune response to TB. The overall aim therefore of this PhD project is to investigate the effect of HCMV on TB risk and mycobacteria-specific antibody responses.

In order to achieve this, the main research objectives are to:

1. Characterise HCMV serostatus in a rural Ugandan cohort (Chapter 3)
2. Measure mycobacteria-specific antibody levels and link these to demographic information and co-infection with HIV and HCMV (Chapter 4)
3. Identify associations between level of HCMV sero-reactivity (and other herpes viruses) and markers of inflammation (Chapter 5)
4. Investigate links between HCMV infection, measures of antibody quality and TB disease risk (Chapter 5)

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CHAPTER 2: MATERIALS, METHODS AND METHOD DEVELOPMENT

2.1 Study Area and Design

The General Population Cohort (GPC) is a population-based open cohort study, set up in 1989 by the Medical Research Council (MRC) UK in collaboration with the Uganda Virus Research Institute (UVRI). Initially established to examine trends in HIV prevalence and incidence, the GPC is located in Kyamulibwa sub-county of Kalungu district, rural south-western Uganda [1]. The cohort now comprises a cluster of 25 neighbouring villages with approximately 20,000 residents (52% aged ≥ 13 years) from three major ethnic groups; the majority (75%) being from the Baganda tribe, the main tribal group in the region. The study population is recruited by an annual house-to-house census. To be eligible for the census, an individual must have spent or be planning to spend at least 3 months in a household within the study area.

Since 2010 research questions have been expanded to include communicable and non-communicable diseases. Long-term epidemiological studies provide evidence for health policy and public health programmes in Uganda and other countries in sub-Saharan Africa. The specific questions asked and the biological samples taken depend on research themes and resources.

Blood samples are taken biannually and transported to MRC/UVRI laboratories in Entebbe where a portion of the venous blood sample is analysed immediately for HIV and other parameters depending on the GPC round protocol. Remaining serum samples are stored at -80°C in a biobank in Entebbe. Most samples used in this PhD were taken from 2011 where vaccination status information was collected, and individuals over the age of 12 years were also investigated for cardiovascular risk factors. Based on a unique identifier, serum samples were linked to demographic information, HIV status and any other information that was collected as part of the annual census.

2.2 Sampling

A total of 2,162 individuals without TB were selected at random, having been stratified by age and sex. Infants under 5 years of age were oversampled in anticipation of high age-dependent HCMV seropositivity based on evidence from other sub-Saharan countries [2]. A target sample size of approximately 100 individuals per year of age from under 1 year to 5 years, and 200 individuals per either 5 or 10 year age groups thereafter. The sex ratio was approximately equal within each age group.

The age structure of sampling for this study was determined based on Ugandan demographics: 49% of Ugandans are under 14 years of age, 21% are between 15-24 years of age, 28% between 25 and 64 years of age and only 2% over 65. The sampling here was similar to the Ugandan demographic with 45% under 14 years, 18% 15-24, 32% 25-64 and 5% over 65 years of age. Samples from TB cases were specifically oversampled and were taken from a range of GPC rounds between 1999 and 2014. Active pulmonary TB was diagnosed through positive sputum smear microscopy after passive case detection. Study participant characteristics are summarized in Table 2-1.

TABLE 2-1: Study participant characteristics (cross sectional study)

Non-TB individuals* (n=2,162)		TB case individuals (n=27)	
Age, years (mean, range)	22.5 (0.08-100.75)	Age, years (mean, range)	36.0 (13.08-56.50)
Sex, percent female (number/total)	50% (1,080/2,162)	Sex, percent female (number/total)	63% (17/27)
HIV prevalence, percentage (number/total), number missing	4.3% (93/2122), 40 unknown HIV status	HIV prevalence, percentage (number/total)	33.3% (9/27)
BCG vaccinated, percentage (number/total), number missing	64.7% (1,371/1,927), 192 unknown BCG status	BCG vaccinated, percentage (number/total), number missing	0% (0/3), 24 unknown BCG status

* Non-TB individuals here were not investigated for active or latent TB.

2.2.1 Power calculation

Based on data from a Phase 2b South African infants vaccine study [3], an increase of approximately 0.5 Optical Density units (OD) in Ag85A IgG was observed in the median response in the control vs case infants. These data indicate that a change of 0.5 OD may be biologically meaningful in terms of protection against TB disease. Using power calculations for a type 1 error (alpha) of 0.05, power of 0.80, with an effect size of 0.5 OD, we would require 64 individuals per comparison group. However, using an estimate from a case control study is likely to result in

differences larger than we would find in the general population. Higher variability is likely to adversely affect the power of the study. Based on an increase in the variance estimate of 20%, the resulting effect size of 0.4 OD would require 100 individuals per group. Since we have a minimum of 100 individuals per comparison group, we are confident that this number will enable us to see differences between groups with 0.4 OD effect size. If individuals are given BCG at birth, we anticipate antibody levels to be rising and remain high up to approximately 5 years (when we know BCG to be effective) and then decline into adolescence and adulthood (where we see most disease). We therefore included approximately 100 children from each age group <1, 1, 2, 3, 4, 5 years (total 600), 200 children aged 6-10, 11-15 and 16-20 (total 600), and 800 people aged 21 years and over. As long as the effect size is equal or greater than 0.4 OD, we expect to have reasonable power to see a change in antibody optical OD between age groups.

2.2.2 TB case samples

Twenty seven individuals with active TB were identified, and all available stored samples were retrieved. These samples were taken at various times prior to, and up to 3 months after, diagnosis. Between 1 and 4 stored serum samples per TB case were identified and retrieved from the biobank of stored samples at time points before, and up to 3 months after TB diagnosis.

All non-TB individual samples and all samples from TB cases were tested for HCMV IgG, mycobacterial antibodies, tetanus toxoid IgG and total IgG. A subset of samples was then selected as a nested 'case-control' sub study for further testing (detailed in Chapter 5). The nested case-control study included 52 samples from 26 of the 27 active TB cases and included matched controls from the GPC (see Chapter 5 for details on sampling).

For the purposes of analysis for Chapters 3 and 4, only one sample per TB case (the sample taken closest to TB diagnosis) was used for cross-sectional analyses of HCMV seropositivity,

mycobacterial and TT antibody and total IgG levels. For Chapter 5, all available samples taken from active TB cases was used.

2.2.3 Sampling of matched controls

Samples from non-TB individuals were selected to be part of a nested case-control sub study (Chapter 5) for further testing. Samples from the 2,162 individuals without active TB were matched to 52 samples from 26 TB cases based on age, sex and HIV status at the point of sampling. Between 4 and 6 control individuals were matched per TB case sample (a maximum of one sample per control. Total number of control individuals – 300). Characteristics of the individuals selected for the nested case-control study are summarized in Table 2-2 below.

Table 2-2: Study participant characteristics (nested case control study – Chapter 5))

	Non TB Control individuals	TB Case individuals	Total
Number of individuals	300	26	326
Mean age (range) yrs	34.5 (2.8-56.5)	36.3 (13.1-56.5)	34.8 (2.8-56.5)
Number female (%)	183 (61%)	15 (58%)	198 (61%)
Number HIV positive (%)	80 (27%)	8 (30%)	88 (27%)

Random sampling without replacement was used based on sex and HIV firstly, and then age was matched as closely as possible. Age at which sample was taken was matched within one year for HIV negative samples however increased variation was seen in HIV positive samples due to a smaller initial pool of HIV positive individuals within the 2,162 sample of non-TB individuals. In

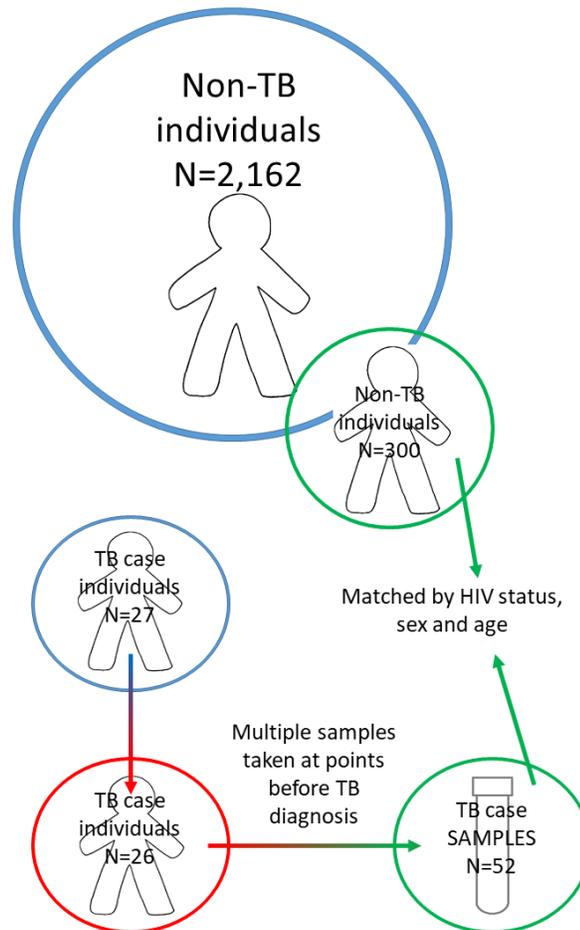
total, 352 samples were included in the nested case-control sub study. We categorized the TB case samples into three time point groupings based on numbers of samples available and time before TB diagnosis: between 14 and 5 years prior to TB diagnosis, between 5 and 1 year prior to TB diagnosis, and between 1 year prior and 3 months after TB diagnosis. Because of the ubiquity of HCMV infection, HCMV seronegative samples were excluded (n=9, all nine were control individuals with a mean age of 37 years (26.9-50.8 years), two of these nine were HIV positive, see Chapter 5).

Table 2-3 details sample numbers included in the case-control sub study.

Table 2-3: TB case sample numbers and time before TB diagnosis

Time before/after TB diagnosis	Non TB Control samples	TB Case samples	Total
14-5 yrs before	99	17	116
5-1 yr before	94	16	110
1 yr before - 3 months after	107	19	126
Total	300	52	352

Figure 2-1 – Sampling for cross sectional (blue) and case control (green) study designs. One TB case individual became HIV positive during sampling and so was excluded from the case control study.



2.2.4 Sampling for MGIA assay

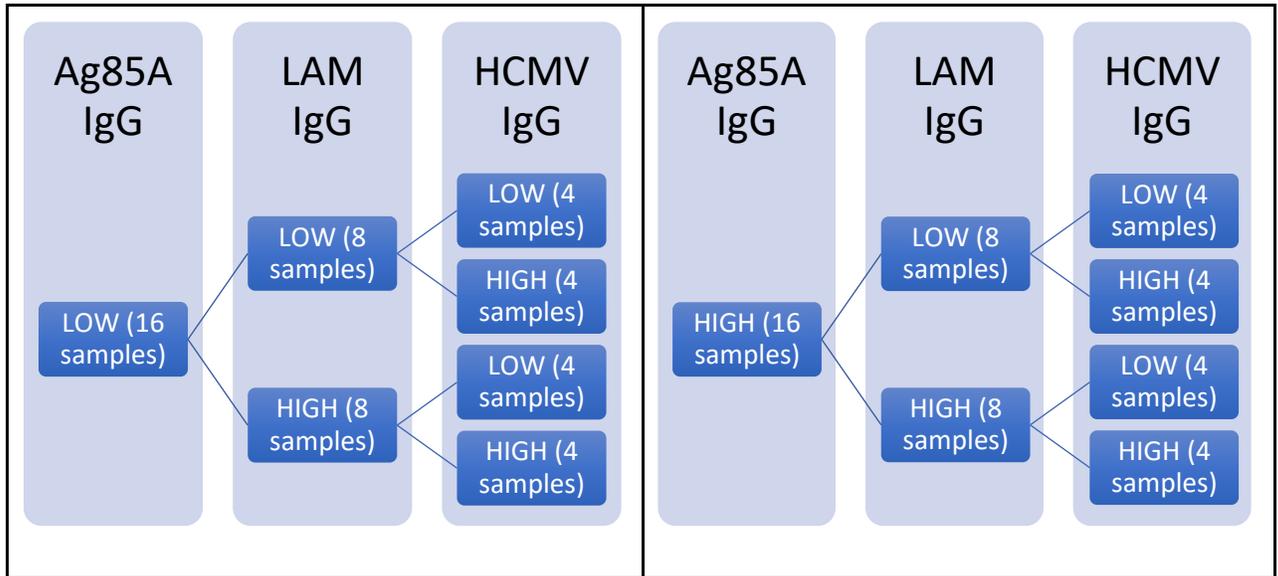
Ex-vivo mycobacterial growth inhibition assay (MGIA) was used to determine effect of serum from the GPC Ugandan cohort. MGIA is used to assess effect of immune cells upon growth of BCG over a 4-day culture period before cells are lysed and bacteria transferred to a tube where bacterial growth is quantified based on a fluorescent quench curve (see methods below). Normally the 4-day culture period uses Fetal Bovine Serum (FBS) as the source of serum. This experiment instead

used human serum from GPC serum samples characterised with respect to their anti-mycobacterial and HCMV antibody content. Controls included FBS, pooled human serum (AB serum) and IgG-depleted AB serum.

Serum samples from the GPC were chosen with respect to their content of certain antibodies hypothesised to be important in TB disease (Ag85A, LAM or HCMV). HIV negative GPC donor serum from individuals over the age of 15 years was randomly selected based on antibody levels. Four samples per group in the upper or lower 20% of antibody levels were chosen.

To ensure that high and low responders for one antibody were controlled for other antibodies, a matrix was constructed (Figure 2-2). This ensured that, for example, all 4 samples that were high Ag85A IgG responders, were not all HCMV IgG high responders. Thirty-two samples were chosen, 16 of which had low Ag85A IgG, 16 had high Ag85A IgG. Each of these 2 groups had 8 low LAM IgG responders and 8 high LAM responders. Within the 8 low or 8high LAM IgG responders, 4 had low HCMV IgG and 4 had high HCMV IgG.

Figure 2-2: Matrix of antibody responses of randomly selected samples for MGIA (sample numbers in parenthesis)



2.3 Ethics

Written consent for the use of clinical records and biological samples for research purposes was obtained from all GPC participants following Uganda National Council of Science and Technology guidelines.

Ethical approval for the use of GPC samples for this study was obtained from The UVRI Research and Ethics Committee and from the Uganda Council for Science and Technology (APPENDIX 1), in addition to the London School of Hygiene & Tropical Medicine (LSHTM), London, UK (APPENDIX 2a). Initially reagents were shipped to UVRI, Entebbe, Uganda and assays were conducted at UVRI laboratories. Subsequently samples were shipped to LSHTM and stored at -80°C until used.

Ethical approval for collection and use of samples from LSHTM volunteers was obtained (APPENDIX 2b).

2.4 Preparation of ELISA standards

Serum from BCG-vaccinated UK donors was tested for IgG to PPD and the highest and lowest responders were pooled to create a stock of standards for use in anti-mycobacterial ELISAs.

2.5 Enzyme-linked immunosorbent assays (ELISAs)

ELISAs were performed to determine Human Cytomegalovirus (HCMV), Epstein Barr Virus (EBV) and Human Simplex Virus (HSV)1/2 serostatus of GPC donors, to quantify the levels of anti-mycobacterial antibodies against PPD, LAM, Ag85A and ESAT6/CFP10, and to measure IgG levels against tetanus toxoid in all GPC donors.

ELISA was also used in combination with a standard curve to quantify total IgG levels, and to measure antibody avidity of PPD IgG antibodies.

Methods pertaining to each assay are detailed below.

2.5.1 Human Cytomegalovirus (HCMV) ELISA

Serum samples were tested for antibodies against HCMV using a standard commercially available ELISA (IBL International, Germany). Testing was conducted at UVRI in Entebbe. Briefly, serum samples were thawed and diluted as per kit instructions to 1:100 in sample diluent. One hundred microliters of diluted serum and controls in duplicate were dispensed into a 96-well plate coated

with HCMV antigen. Plates were covered and incubated for 1hr at 37°C and then washed before 100µL of anti-IgG conjugate was added and the plate covered and incubated again for 30 minutes at room temperature. After another washing step, 100µL of tetra-methyl-benzidine (TMB) substrate solution was added and the plate incubated for 15 minutes in the dark at room temperature before 100µL of 0.2M sulphuric acid solution was added to stop the reaction. Plates were read at 450/620nm within 30 minutes.

Individuals were considered to be seropositive if the IgG OD measurement of plate controls fell within the kit specifications and the mean of duplicate measurements was above the calculated cut off.

2.5.2 Epstein-Barr virus (EBV) ELISA

EBV is the etiological agent of infectious mononucleosis. A subset of serum samples was tested for IgG against Epstein-Barr virus nuclear antigen 1 (EBNA-1) using a commercial kit (Euroimmun, Germany). Testing was conducted at LSHTM, UK. As per kit protocol, all reagents were brought to room temperature and 100µL of calibrator samples, positive and negative controls along with 1:100 diluted test samples were aliquotted into appropriate wells to extrapolate fully quantitative measurements. Samples were incubated for 30 minutes at room temperature before plates were washed 3 times. One hundred microliters of HRP-conjugated anti-human IgG was then added to each well and the plates incubated for another 30 minutes at room temperature. Following another 3 washes, 100µL of chromogen substrate was added, the plates incubated for 15 minutes at room temperature in the dark and 100µL of stop solution added. Plates were read at 450nm within 30 minutes of the addition of the stop solution.

The resulting measurement (in relative units (RU)) was calculated based on a standard curve from the calibration sera and, based on kit cut-offs, samples were reported as positive or negative.

2.5.3 Herpes Simplex Virus (HSV) 1/2 ELISA

This commercial kit (Euroimmun, Germany) uses a mixture of HSV1 and HSV2 full antigens to detect IgG specific to both subtypes. Testing was conducted at LSHTM, UK. As per kit protocol, all reagents were brought to room temperature and 100µL of calibrator samples, positive and negative controls, along with 1:100 diluted test samples were aliquotted into appropriate wells in order to extrapolate fully quantitative measurements. Samples were incubated for 30 minutes at room temperature before plates were washed 3 times. One hundred microliters of HRP-conjugated anti-human IgG was then added to each well and the plates incubated for another 30 minutes at room temperature. Following another 3 washes, 100µL of chromogen substrate was added, the plates incubated for 15 minutes at room temperature in the dark and 100µL of stop solution added. Plates were read at 450nm within 30 minutes of the addition of the stop solution. The resulting measurement (in relative units (RU)) was calculated based on a standard curve from the calibration sera and, based on kit cut-offs, samples were reported as positive or negative.

2.5.4 Mycobacterial ELISA method development

Ag85A IgG ELISA methodology was transferred from Aeras (MD, USA) and method development was conducted on other mycobacterial antigens used in this study to determine the appropriate concentration of coating antigen and detection antibody. Serum titrations were performed to determine the best dilution of serum for the assays.

Due to the small volume of sera from Ugandan GPC donors, method development was conducted using sera from UK volunteers and confirmed in randomly selected GPC samples. In addition, the

use of half volume ELISA plates was investigated to reduce the volume of serum needed to run the assays.

A serum dilution of 1:100 was determined to give the largest range of OD readings of the spectrophotometer for all antigens. Dilutions of 1:500 for the IgG secondary antibody, and 1:10,000 for the IgM secondary antibody were used. Half volume ELISA plates (Fisher Scientific) gave similar results as full volume and so half volume plates were used for all ELISAs in this project.

Concentrations of coating antibodies are detailed in the mycobacterial ELISA section below. Coating antibodies were not fully titrated but based upon a concentration that gave good differentiation in serum titration experiments.

ELISA worksheets were used for all experiments. Times and dates of experiments and addition of reagents, as well as incubation times were noted to ensure ELISA plates run on different dates were comparable. An example of a worksheet can be found in APPENDIX 3.

2.5.5 Mycobacterial ELISA

Antibody content was measured using an ELISA. Testing was conducted at UVRI in Entebbe, Uganda (Ag85A IgG and PPD IgG) and LSHTM, London, UK (CFP10/ESAT6 IgG, Ag85A IgM and LAM IgG). Half volume ELISA plates (Fisher Scientific) were coated overnight at 4°C with 50µL sodium carbonate buffer containing 1.5µg/mL recombinant Ag85A protein (Aeras, USA), 1.5µg/mL PPD (Lot 051815KA, Aeras, USA), 0.5µg/mL LAM (NR-14848; BEI Resources, VA, USA), 0.25µg/mL each CFP10 (NR-49425; BEI Resources, VA, USA) and ESAT6 (NR-14868; BEI Resources, VA, USA). Plates and reagents were brought to room temperature and the plate washed 3 times with PBS, 5% Tween20 (v/v) (PBST). The plate was blocked for 1 hour at room temperature with 100µL blocking buffer (PBS, 5% milk (w/v)). Serum was diluted 1:100 with PBS, 5% milk (w/v) and 50µL of each

sample in duplicate was added to the appropriate wells in the ELISA plate. High and low PPD responder control samples were prepared at the same dilution as the test samples, and controls and plate blanks (consisting of assay diluent alone with no serum) were added in triplicate. The plate was sealed and incubated for 2 hours at room temperature. Plates were then washed five times with PBST and 50µL of an appropriately diluted horseradish-peroxidase (HRP)-conjugated secondary antibody in PBST-5% milk: for measurement of IgG; 1:500 dilution of goat anti-human IgG-conjugated to HRP (04-10-20; KPL, Gaithersburg, MD, USA), for measurement of IgM; 1:10,000 dilution of goat anti-human IgM-conjugated to HRP (ab97205; Abcam) was added to each well for one hour at room temperature. The plate was washed seven times with PBST and 50µL tetramethylbenzidine (TMB, BD BioSciences, San Jose, CA, USA) was added to each well. The plate was incubated for 15 minutes in the dark before the reaction was stopped by adding 50µL of 2M Sulphuric Acid (Sigma, St. Louis, MO, USA) to each well. Absorbance was measured using a microplate reader at 450nm within 30 minutes to obtain optical density (OD), a surrogate marker of antibody titre when the assay is conducted on the linear part of the curve.

Mean blank values (wells coated with antigen and secondary conjugated antibody but no serum) were subtracted from all OD readings. Geometric means were used in order to reduce the effect of skewed distribution as has been recommended in immunoepidemiological studies [5].

2.5.6 Tetanus toxoid ELISA

IgG specific to TT was measured at LSHTM, UK by ELISA. Half volume ELISA plates (Fisher Scientific) were coated overnight at 4°C with 50µL sodium carbonate buffer containing 0.5µg/mL Tetanus Toxoid (02/232, NIBSC, Potters Bar, UK). Plates and reagents were brought to room temperature and the plate washed 3 times with PBS, 5% Tween20 (v/v) (PBST). The plate was blocked for 1 hour at room temperature with 100µL blocking buffer (PBS, 5% milk (w/v)). Test sera were diluted 1:100

with PBS, 5% milk (w/v) and 50µL of each sample in duplicate was added to the appropriate wells in the ELISA plate. Control serum (from vaccinated UK donor pooled sera) was also diluted 1:100. Controls and plate blanks consisting of assay diluent alone with no serum were added in triplicate. The plate was sealed and incubated for 2 hours at room temperature. It was then washed five times with PBST and 50µL of goat anti-human IgG- conjugated to HRP (04-10-20; KPL, Gaithersburg, MD, USA) diluted 1:500 in PBST-5% milk was added to each well for one hour at room temperature. The plate was washed seven times with PBST and 50µL tetramethylbenzidine (TMB, BD BioSciences, San Jose, CA, USA) was added to each well. The plate was incubated for 15 minutes in the dark before the reaction was stopped by adding 50uL of 2M Sulphuric Acid (Sigma, St. Louis, MO, USA) to each well. Absorbance was measured using a microplate reader at 450nm within 30 minutes to obtain optical density (OD), a surrogate marker of antibody titre.

As above, mean blank values (wells coated with antigen and secondary conjugated antibody but no serum) were subtracted from all OD readings and geometric mean used in analysis.

2.5.7 Total IgG ELISA method development

In order to find the dynamic range of IgG standards to use, a full titration curve was conducted using one GPC serum sample (sample number 910163), pooled UK serum control, and an IgG antibody standard (I4506; Sigma). The intention was to find approximately five concentrations of the IgG standard that would form a reproducible standard curve to be used on each plate with which to extrapolate total IgG concentration in test sera.

A doubling dilution was used to obtain a titration from 1:100 to 1: 13,421,772,800 of IgG standard which, based on an initial concentration of 0.86mg/mL of IgG, equates to concentrations ranging from 8.6µg/mL to 16.8ng/mL (see Table 2-4).

Two concentrations of mouse anti-human IgG coating antibody (ab 200699; Abcam) were trialled (1.0 and 0.5µg/mL) and a concentration of 0.5µg/mL was determined to give similar OD measurements as the higher dilution but with minimal background. Duplicate samples of each dilution of standard and test sample were plated following an overnight incubation of 0.5µg/mL overnight at 4°C with 50µL sodium carbonate buffer containing mouse anti-human IgG at 0.5ug/mL

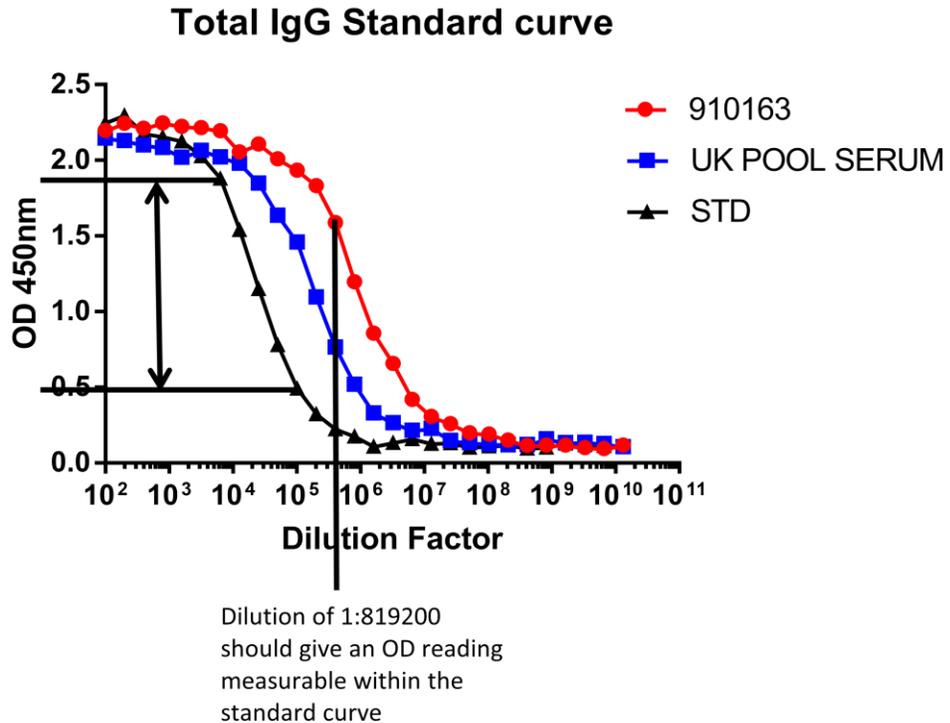
Table 2-4 – IgG standard curve titration

Standard dilution	mg/mL	ug/mL	ng/mL
100	0.0086	8.6	8600
200	0.0043	4.3	4300
400	0.00215	2.15	2150
800	0.001075	1.075	1075
1600	0.0005375	0.5375	537.5
3200	0.00026875	0.26875	268.75
6400	0.000134375	0.134375	134.375
12800	6.71875E-05	0.0671875	67.1875
25600	3.35938E-05	0.03359375	33.59375
51200	1.67969E-05	0.016796875	16.79688
102400	8.39844E-06	0.008398438	8.398438
204800	4.19922E-06	0.004199219	4.199219
409600	2.09961E-06	0.002099609	2.099609
819200	1.0498E-06	0.001049805	1.049805
1638400	5.24902E-07	0.000524902	0.524902
3276800	2.62451E-07	0.000262451	0.262451
6553600	1.31226E-07	0.000131226	0.131226
13107200	6.56128E-08	6.56128E-05	0.065613
26214400	3.28064E-08	3.28064E-05	0.032806
52428800	1.64032E-08	1.64032E-05	0.016403

104857600	8.2016E-09	8.2016E-06	0.008202
209715200	4.1008E-09	4.1008E-06	0.004101
419430400	2.0504E-09	2.0504E-06	0.00205
838860800	1.0252E-09	1.0252E-06	0.001025
1677721600	5.126E-10	5.126E-07	0.000513
3355443200	2.563E-10	2.563E-07	0.000256
6710886400	1.2815E-10	1.2815E-07	0.000128
13421772800	6.4075E-11	6.4075E-08	6.41E-05

Figure 2-3 shows the dynamic range of standards and samples. For standards, dilutions of 1:6,400, 1:12,800, 1:25,600, 1:51,200 and 1:102,400 (with respective concentrations of 134.4, 67.2, 33.6, 16.8 and 8.4ng/mL of IgG – highlighted in grey in Table 2-4) were chosen as being on the linear portion of the curve – highlighted on the graph. For the test sera, a dilution of approximately 1:819,200 gives an OD reading within the dynamic range of the spectrophotometer used to read plates (between 0.5 and 1.6 OD), therefore a dilution of 8×10^5 was chosen for all test sera.

Figure 2-3- Standard curve for IgG and test sera titrations (UK pooled sera and one GPC sample)



2.5.8 Total IgG ELISA

All samples were tested for total IgG content at LSHTM, London, UK. Half volume ELISA plates (Fisher Scientific) were coated overnight at 4°C with 50µL sodium carbonate buffer containing mouse anti-human IgG at 0.5µg/mL (ab 200699; Abcam). IgG antibody standards (134.4-8.4 ng/mL) were prepared by diluting purified human IgG (0.86 mg/mL; I4506; Sigma) in PSBT-5% milk and test sera were diluted 8x10⁵ in PSBT-5% milk. Plates and reagents were brought to room temperature and the plate washed 3 times with PBST. The plate was blocked for 1 hour at room temperature with 100µL blocking buffer (PBS, 5% milk (w/v)). Standards and diluted test samples were aliquotted in duplicate and the sealed plate was incubated for one hour at 37°C. The plate

was washed 5 times with PBST and 50ul of HRP-conjugated goat anti-human Fc (ab97225; Abcam) diluted 1/500 with PBST-5% milk was added for 1 hour at room temperature. As above, plates were developed using TMB, and sulphuric acid was used to stop the reaction. Plates were read at 450nm and OD measurements converted into g/L by use of the standard curve on each plate (calculations detailed in APPENDIX 4).

2.6 Luminex

Luminex multiplex cytokine platform was used to determine the concentrations of IL-6, TNF- α , IFN- γ , IFN- α 1, IL-1a, IP-10, IL-1b, IL-1Ra, IL-10, IL-12p40, IL-12p70. Cytokines were tested using Millipore kits (Merck Millipore, USA) according to the manufacturer's instructions. Standard curves were generated from the serial dilutions that were made from the assay controls supplied and matched against the cytokine concentration for quantification. The concentrations of all the analytes in the quality control reagents were found to be within the ranges expected. The Bio-Plex manager version 6.1 was used for bead acquisition and analysis of median fluorescence intensity (MFI).

2.7 Sodium thiocyanate antibody avidity

Antibody avidity was assessed by ELISA using the chaotropic agent sodium thiocyanate (NaSCN) to dissociate low-affinity IgG binding using a previously described method [6]. An avidity index (AI) was calculated as the molar concentration of NaSCN required to reduce antigen specific IgG binding by 50%.

An 8M stock solution of NaSCN (Acros Organics, Belgium) in water was prepared (19.45g NaSCN in 30ml water). Further concentrations were prepared from the 8M stock to give eight concentrations between 0-7M NaSCN.

All samples were tested for antibody avidity to PPD at LSHTM, London, UK. Half volume ELISA plates (Fisher Scientific) were coated overnight at 4°C with 50µL sodium carbonate buffer containing 1.5ug/mL PPD (Lot 051815KA, Aeras, USA).

Plates and reagents were brought to room temperature and the plate washed 3 times with PBST. The plate was blocked for 1 hour at room temperature with 100µL blocking buffer (PBS, 5% milk (w/v)). Serum was diluted 1:100 with PBS, 5% milk (w/v) and 50µL of each sample in duplicate for each NaSCN concentration was added to the appropriate wells in the ELISA plate (Figure 2-4).

The plate was sealed and incubated at room temperature for 2 hours. Without removing the sample, 50µL of NaSCN of the appropriate dilution was added. The plate was incubated at room temperature for 15 minutes before the plates were washed 5 times with PBST.

50 µL of 1:500 dilution goat anti-human IgG-conjugated to HRP detection antibody (04-10-20; KPL, Gaithersburg, MD, USA) was then added to each well, the plate sealed again and incubated at room temperature for 1 hour. The detection antibody was removed and the plate washed 7 times with PBST and 50µL tetramethylbenzidine (TMB, BD BioSciences, San Jose, CA, USA) was added to each well. The plate was incubated for 15 minutes in the dark before the reaction was stopped by adding 50uL of 2M Sulphuric Acid (Sigma, St. Louis, MO, USA) to each well. Absorbance was measured using a microplate reader at 450nm within 30 minutes to obtain optical density (OD) which was then converted into AI using calculations detailed in APPENDIX 5.

Figure 2-4 – 96 well plate layout of sodium thiocyanate avidity assay. Duplicate samples are plated for each concentration of NaSCN (S1 – Sample 1)

Conc of NaSCN	1	2	3	4	5	6	7	8	9	10	11	12
0	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
1M	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
2M	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
3M	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
4M	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
5M	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
6M	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6
7M	S1	S1	S2	S2	S3	S3	S4	S4	S5	S5	S6	S6

2.8 Peripheral Blood Mononuclear Cells (PBMC) isolation

To isolate PBMC, 15mL Lymphoprep (Axis Shield) was added to Leucosep tubes (Greiner) and centrifuged at 1800rpm for 1 minute to pull the lymphoprep below the level of the porous disc.

Between 15-30mL of heparinised blood was added to each leucosep tube and tubes centrifuged at 1000 x g for 13 minutes at room temperature. The centrifuge was left to stop on its own, without the use of a brake. The plasma fraction was removed and the PBMC at the interface were collected into a falcon tube (Fisher Scientific), with R0 (RPMI (Sigma), 1% Penicillin-Streptomycin (Gibco), 1% L-Glutamine (Gibco)) added, up to a volume of 45ml. Tubes were centrifuged at 1800rpm at room temperature for 5 minutes, with braking. The supernatant was discarded and the pellet resuspended in 45ml R0 before centrifuging again at 1800 rpm at room temperature for 5 minutes. Cells were resuspended in 10mL R10 (RPMI (Sigma) 10% FCS (Biosera), 1% Penicillin-Streptomycin (Gibco), 1% L-Glutamine (Gibco), 1% Sodium Pyruvate (Gibco) for counting.

2.9 Ex-vivo IFN- γ Enzyme-Linked ImmunoSpot (ELISPOT)

ELISPOT is used to detect and enumerate cytokine-secreting antigen-specific T-cells. Here, IFN- γ secretion was measured. ELISPOT plates (Millipore) were prepared by adding 50 μ L of 0.05M carbonate-bicarbonate buffer containing 15 μ g/mL anti-IFN- γ capture antibody (Mabtech) to each well and incubating overnight at 4°C. The plate was washed 5 times with 120 μ L per well of sterile PBS and 100 μ L of R10 blocking solution added to each well and incubated at 37°C for 2-5 hours. Following blocking, 0.3x10⁶ PBMC in a volume of 80 μ L were added to duplicate wells. 20 μ L of each antigen was then added to the appropriate wells (each antigen was tested in duplicate). Antigens were prepared as follows: PPD (FDA) 100 μ g/mL to give a final concentration of 20 μ g/mL, phytohemagglutinin (PHA, Sigma) and phorbol 12-myristate 13-acetate (PMA, Sigma) which are potent positive controls, at 50 μ g/mL and 250 μ g/mL respectively to give a final concentration of 10 μ g/mL and 50 μ g/mL respectively, Antigen85B (Ag85B, BEI resources) 50 μ g/mL to give a final concentration of 10 μ g/mL, lipoarabinomannan (LAM, BEI Resources) 100 μ g/mL to give a final

concentration of 20µg/mL and a mixture of ESAT6 and CFP10 (both BEI resources) at 50µg/mL (25µg/mL each antigen) to give a final concentration of 10µg/mL (5µg/mL each antigen).

The plate negative control was 20µL of R10. The plate was then incubated for 18-20 hours at 37°C before washing 5 times with 1xPBS w/0.05% Tween20 (v/v). Biotin anti-IFN-γ antibody (Mabtech) was diluted 1:1000 with PBS and 50µL added to each well. The plate was incubated for 2 hours at room temperature before washing 5 times with PBS as above. Streptavidin-ALP (alkaline phosphatase) was diluted 1:1000 in PBS and 50µL was added to each well. The plate was incubated for 1 hour at room temperature, washed 5 times and 50µL BCIP/NBT (Europa Bioproducts) developer was then added to each well. Colour was left to develop for 5 minutes and the reaction was terminated by running the plate under tap water. The plate was allowed to dry on the bench overnight before reading the next day using a plate reader and ELISPOT 5.0 software (AID Diagnostika, Germany).

2.10 Mycobacterial Growth Inhibition Assay (MGIA)

MGIA was conducted by modifying the Zelmer *et al* protocol [7]. MGIA is used to assess effect of immune cells upon growth of BCG (used as a surrogate for *M.tb*) over a 4 day culture period. After culture, cells are lysed and bacteria transferred to a tube where bacterial growth is quantified based on a fluorescent quench curve. Normally the 4 day culture period uses Fetal Bovine Serum (FBS) as the source of serum. This experiment instead used human serum from GPC serum samples characterised with respect to their anti-mycobacterial or HCMV antibody content. Sera from 5 TB cases was also included (see sampling methods, above). Controls (in duplicate) included FBS (Biosera), heat inactivated pooled human serum (AB serum, Sigma) and IgG-depleted AB serum.

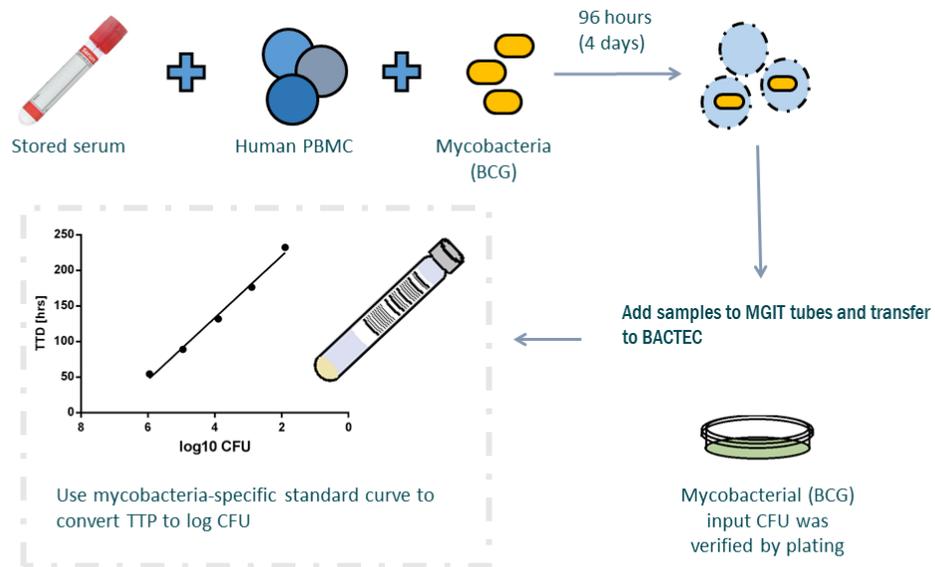
The BD MGIA system is based on fluorescence quenching by oxygen. During bacterial growth, oxygen levels are depleted, releasing fluorescence which is measured hourly by the BACTEC system. A standard curve is produced with known bacterial concentrations to convert a Time To Positivity (TTP) readout into a Colony Forming Unit (CFU) measurement. In these experiments, fresh human PBMC from one UK-born LSHTM volunteer who was not historically vaccinated with BCG was used for the MGIA experiments. Figure 2-5 illustrates the methodology.

Following isolation (see methods above), PBMC were resuspended in RPMI-MGIT (RPMI-1640 HEPES modification (Sigma), 2mM L-Glutamine (Gibco), containing no FCS) at a concentration of $14.6 \times 10^6/\text{mL}$ to give a final concentration of 3.5×10^6 human PBMC in 240 μL . 60 μL of each test sera was added to the PBMC. 300 CFU/mL BCG was prepared in RPMI-MGIT to give a final input dose of 90 CFU in 300 μL . Human PBMC, test sera and BCG were co-cultured for 4 days in a 37°C, 5% CO₂ incubator in 48-well plates where the outer-most wells were filled with 1mL RPMI-MGIT to minimise evaporation from test wells. BCG input CFU was verified by plating 100 μL in duplicate onto 7H11 agar plates and counting colonies after approximately 2-3 weeks of incubation at 37°C.

After 4 days of incubation, 48-well plates were removed from the incubator and centrifuged at 12,000rpm for 10 minutes. 500 μL of supernatant was removed, ensuring the pellet remained intact. 400 μL of sterile tissue grade water was added to lyse the cells, and liquid in the plates was mixed by pipetting. After standing at room temperature for 5 minutes, liquid in the wells was again mixed by pipetting. 800 μL MGIT PANTA (BD) enrichment medium was added to each MGIT tube (BD) and then the total volume of one well (approximately 500 μL) was added to the MGIT tube. After inverting the tube to mix the contents, the tube was placed in the BACTEC MGIT machine to await a TTP readout. Two “direct-to-MGIT” tubes, containing BCG input only, were included as quality controls to ensure reproducibility across experiments. Based on the initial input

of BCG, the TTP readout was converted to CFU using a previously prepared standard curve in GraphPad Prism 7 software.

Figure 2-5 – MGIA methodology. Adapted with permission from H. Painter.



2.11 Statistical analysis

Data were entered using Microsoft Excel and analysed using Stata version 14 (Stata Corporation, College Station, TX, USA). Some method development work and MGIA results were analysed using GraphPad Prism 7.

Due to the large number of different exposures investigated in this project and the possibility of multiplicity, a p value of 0.01 was considered strong evidence against the null hypothesis of no difference between comparison groups, and 99% confidence intervals (CI) are reported.

A variety of statistical analyses were used and further details are given in corresponding results sections.

2.13 References

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CHAPTER 3 - RESEARCH PAPER

Human cytomegalovirus epidemiology and relationship to tuberculosis in a rural Ugandan cohort

3.1 Chapter background

This chapter is a research paper published in PLOS One [1] with the title “Human cytomegalovirus epidemiology and relationship to cardiovascular and tuberculosis disease risk factors in a rural Ugandan cohort” (see Appendix 6). This chapter fulfills aim 1: to characterise HCMV serostatus in the cohort. This paper measures HCMV seropositivity in the GPC and explores magnitude of HCMV seropositivity (measured by IgG) and associations with age, sex, TB and HIV.

The published research paper also utilizes the serendipitous availability of CVD risk factor data for adults within the cohort. This enabled investigation into associations between HCMV response and CVD, a relationship seen in high-income countries but not studied in low-income settings. For the purposes of this thesis, however, only those sections pertaining to the PhD project aims are included in this chapter.

3.2 Abstract

Human cytomegalovirus (HCMV) infection has been associated with increased morbidity and mortality in high-income countries (HICs). There is a paucity of data in low- and middle-income countries (LMICs) where HCMV seropositivity is higher. Serum samples from 2,174 Ugandan individuals were investigated for HCMV antibodies and data linked to demographic information and HIV and TB co-infections. HCMV seropositivity was 83% by one year of age, increasing to 95% by five years. Female sex, HIV positivity and active pulmonary tuberculosis

(TB) were associated with an increase in HCMV IgG levels in adjusted analyses. HCMV infection is ubiquitous in this rural Ugandan cohort from a young age. The association between TB disease and high HCMV IgG levels merits further research.

3.3 Introduction

Human Cytomegalovirus (HCMV), also known as human herpesvirus-5 (HHV-5), is a member of the β -herpesviridae subfamily which is widely distributed in human populations. HCMV transmission occurs through person-to-person contact. It can be transmitted transplacentally to neonates or through breast milk of an infected and shedding mother, by intimate contact and by transplantation from (or sharing syringes with) an infected individual [2]. It has been shown that young children shed HCMV virus in saliva and urine at high levels which may add to transmission between infants and adult caregivers [3].

Congenital HCMV infection is the leading cause of permanent hearing and neurological impairment as well as vision loss in infants worldwide [4]. Maternal primary infection, re-infection, or reactivation, especially during the first trimester, is particularly associated with adverse neonatal outcomes [5]. The incidence of congenital HCMV infection is estimated at between 0.7 and 5% of all births in low- and middle-income countries (LMICs) [6].

In immunocompetent adults, HCMV infection rarely causes disease; however, once infected, the virus remains latent in a wide range of cell types, including lymphocytes and myeloid lineage cells, as well as smooth muscle cells and endothelial cells which line blood vessels [7]. HCMV/HIV co-infection is common and is an important cause of HCMV retinitis and severe non-AIDS events, including death, in HIV-infected individuals [8,9]. HCMV infection is associated with chronic immune activation [10] and recent evidence implicates immune activation with increased risk of tuberculosis (TB) disease [11].

In this study, we investigate HCMV seroprevalence in a large cross-sectional rural Ugandan cohort (n=2,174) and investigate associations with co-infections and demographic information.

3.4 Material and Methods

3.4.1 Study Area and design

The General Population Cohort (GPC) is a population-based open cohort study, set up in 1990 by the Medical Research Council (MRC) UK in collaboration with the Uganda Virus Research Institute (UVRI). Initially established to examine trends in HIV prevalence and incidence, the GPC is located in Kyamulibwa sub-county of Kalungu district, rural south-western Uganda [12]. The cohort now comprises a cluster of 25 neighboring villages with approximately 20,000 residents (52% aged ≥ 13 years) from three ethnic groups, the majority (75%) being from the Baganda tribe, the main tribal group in the region.

Blood samples are transported to MRC/UVRI laboratories in Entebbe where a portion of the venous blood sample is analysed according to protocol guidelines. Remaining samples are stored at -80°C in a biobank in Entebbe [12].

The majority of samples tested for HCMV in this study were taken from 2011. Samples from TB cases were from a range of GPC rounds sampled between 1999 and 2014. Active pulmonary TB was diagnosed through positive sputum smear microscopy after passive case detection.

3.4.2 Sampling

Individuals were selected at random, having been stratified by age and sex. Infants under 5 years of age were oversampled in anticipation of high age-dependent HCMV seropositivity based on evidence from other sub-Saharan countries. A target sample size of approximately 100 individuals per year of age from under 1 year to 5 years were sampled and 200 individuals

per 5 or 10 year age groups thereafter. The sex ratio was approximately equal within each age group.

The age structure of sampling for this study was determined based on Ugandan demographics: 49% of Ugandans are under 14 years of age; 21% are between 15-24 years of age; 28% between 25 and 64 years of age and only 2% over 65. The sampling here was similar to the Ugandan demographic with 45% under 14 years, 18% 15-24, 32% 25-64 and 5% over 65 years of age.

Being a cross-sectional study, each individual was only sampled once. Siblings and parent-child pairs were not excluded.

Table 3-1. General Population Cohort characteristics of individuals included in this study

Age, mean (range)	22.7 (0.08-100.75)
HIV prevalence, percentage (number/total)	4.6% (100/2,134)
Sex, percentage female (number/total)	50.2% (1,091/2,174)
Active TB cases, percentage (number/total)	1.3% (27/2,134)
Tribe, percentage (number/total)	
- Baganda	75% (1,524/2,035)
- Rwandese Ugandan	16% (319/2,035)
- Other §	9% (192/2,035)

§ Other tribes include Bakiga, Batooro, Banyankole, Basoga, Bafumbira, Tanzanian, Barundi

3.4.3 Ethics

Written consent for the use of clinical records and biological samples for research purposes was obtained from all GPC participants following Uganda National Council of Science and Technology guidelines.

Ethical approval for the use of samples for this study was obtained from The UVRI Research and Ethics Committee and from the Uganda Council for Science and Technology, in addition to the London School of Hygiene & Tropical Medicine, London, UK.

3.4.4 HCMV serology

Serum samples were tested for antibodies against HCMV using standard, validated and commercially available enzyme-linked immunosorbent assay (ELISA) against HCMV IgG (IBL International GmbH). Testing was conducted at UVRI in Entebbe. Briefly, serum samples were thawed and diluted as per kit instructions to 1:100 in sample diluent. One hundred microliters of diluted serum and controls in duplicate were dispensed into a 96-well plate coated with HCMV antigen. Plates were covered and incubated for 1hr at 37°C and then washed before 100µL of anti-IgG conjugate was added and the plate covered and incubated again for 30 minutes at room temperature. After another washing step, 100µL of tetra-methyl-benzidine (TMB) substrate solution was added and the plate incubated for 15 minutes in the dark at room temperature before 100µL of 0.2M sulphuric acid solution was added to stop the reaction. Plates were read at 450/620nm within 30 minutes to obtain optical density (OD), a surrogate marker of antibody titer.

Individuals were considered to be seropositive if the IgG OD measurement of plate controls fell within the kit specifications and the mean of duplicate measurements was above the calculated cut off.

3.4.5 Linking to GPC data

Testing for HIV was carried out immediately after blood collection in Uganda as previously described by Asiki *et al* [12]. Data were linked to the samples retrieved via each participant's unique GPC identifier. Total IgG was measured by ELISA utilizing a commercial standard curve. Degraded samples with negative total IgG were excluded from the study.

Samples used in this study were linked to demographic and other clinical data corresponding to the same time period at which the sample was taken.

3.4.6 Statistical analysis

Participants were categorized as either HCMV seronegative or seropositive based upon OD results; seropositive samples were further categorized into tertiles of OD, to differentiate low, medium and high responders. The Student's t test was used to compare continuous variables (such as HCMV IgG antibody OD measurement) between two independent groups (sex, tribe (Baganda or other), HIV and TB status). Associations of HCMV IgG OD with age, tribe, sex, HIV and TB were investigated. An analysis of variance (ANOVA) was used to compare continuous variable outcomes between HCMV tertiles.

Linear regression was used to determine the relationship between HCMV IgG OD (as the dependent variable) and age, sex, HIV and TB status (as the independent variables – tribe was excluded in HCMV IgG OD regression analysis due to missing data – 10/27 active TB cases did not have information on tribe). HCMV OD data were slightly positively skewed, possibly resulting in artificially narrower confidence intervals (CI) around estimates.

To account for evidence of non-linearity of HCMV OD with age (likelihood ratio test $p=0.0025$), a quadratic term for age was included in the regression analyses.

To account for multiple comparisons, 99% CI are reported and a p value of 0.01 is considered to represent strong evidence to reject the null hypothesis.

Data were entered using Microsoft Excel and analysed using STATA version 14 (Stata Corporation, College Station, TX, USA).

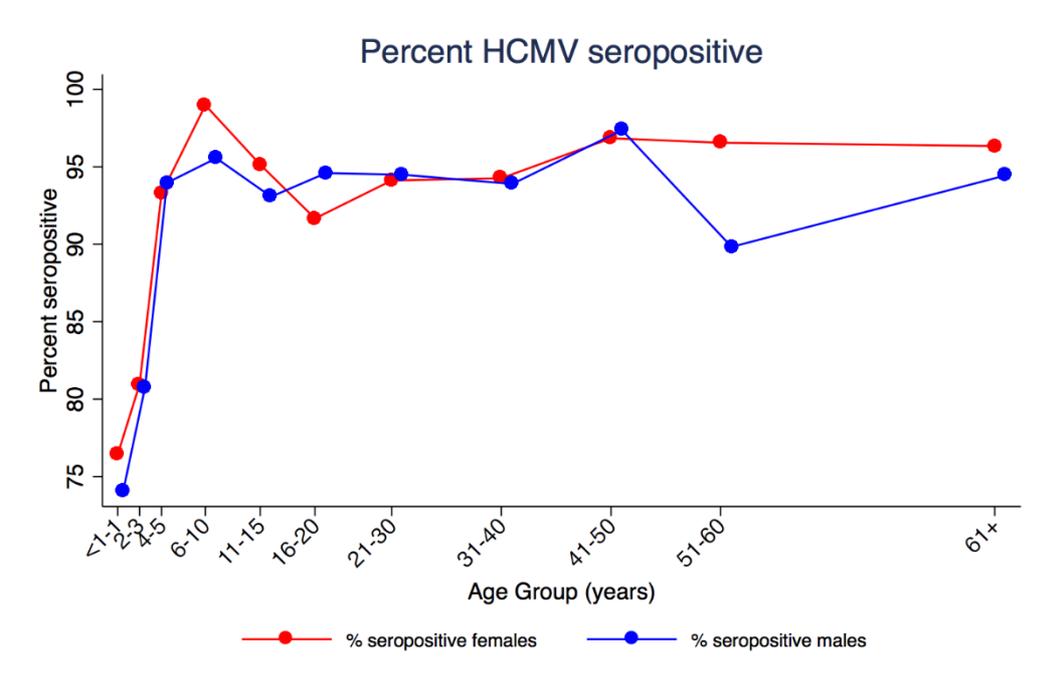
3.5 Results

Of the 2,189 samples tested, 15 had negative total IgG levels and so were excluded due to probable degradation of sample, resulting in 2,174 samples being included in initial analyses. Following these exclusions, 40 individuals did not have a valid HIV result and so were excluded from regression analysis where HIV was included as a regression term.

Overall 91% (1,988/2,174) of the population tested positive for HCMV antibodies. Among children less than 15 years of age, 1% (10/991) were HIV positive; among individuals over 15 years of age, 8% (90/1,143) were HIV positive. The highest proportion infected with HIV (15%) was within the 41-50 year age group.

The percentage of HCMV seropositive individuals in this population increases for both males and females equally from birth until the 6-10 year age group, after which seropositivity levels plateau at around 95% (Figure 1): 83% HCMV seropositivity was seen by age one year and this increased to 95% by age five.

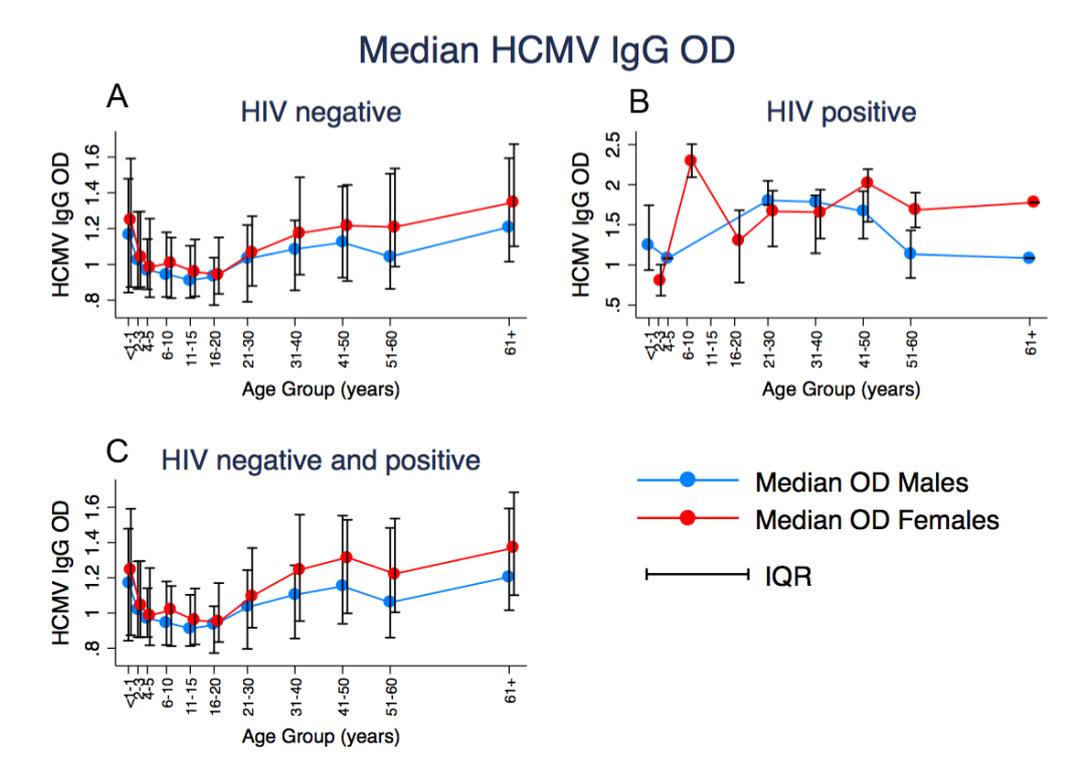
Figure 3-1. Percent HCMV seropositive by age group (years). Red shows females and blue shows males.



Because of the ubiquity of HCMV infection within this population it was decided to conduct further analyses on the HCMV seropositive population only. An analysis of the HCMV negative population (n=186, none of whom were TB cases and only 4 HIV positive) showed no significant associations for sex or HIV in regression analysis. The only significant association was with age in years ($p < 0.001$).

Of the HCMV seropositive individuals, IgG levels initially decrease from a peak in those <1 year until the 16-20 year age group, after which HCMV IgG levels increase for both males and females (Figure 3-2A). When HIV negative individuals are plotted separately, the increased HCMV IgG levels in the 31-50 year old groups coincide with the highest HIV positivity (Figure 3-2B).

Figure 3-2A. Median HCMV IgG OD levels (among HCMV seropositive) in HIV negative individuals by age group (years) and sex. Figure 3-2B. Median HCMV IgG OD levels (among HCMV seropositive) in HIV positive individuals by age group (years) and sex. Figure 3-2C. Median HCMV IgG OD levels in HIV negative and HIV positive individuals combined, HCMV seropositive individuals by age group (years) and sex.



Red lines show female median values, blue lines show male median values. Vertical lines show interquartile range. HCMV - human cytomegalovirus, HIV - human immunodeficiency virus.

To investigate impact of age on HCMV IgG levels, ages were further split into < 9 months, 9 months to 12 years, 13-20 years, 20-60 and 60 years and above. Age groups selected are based on presence of maternal antibodies (conservative estimate of 9months [13]), prior to sexual debut (9 months - 12 years [14]), post sexual debut (13-20 years [14]), 21-59 years and post 60 years. It was found that infants under the age of 9 months have higher mean HCMV IgG (1.25

OD) than any other age group apart from the over 60 years olds (1.34 OD) in unadjusted analyses.

A 0.07 OD increase in HCMV IgG (99% CI 0.02,0.12, $p < 0.001$) is associated with being female in unadjusted analyses. This increase remained after adjusting for age, HIV and TB infection status (mean difference 0.07 OD 99% CI 0.03,0.11, $p < 0.001$) (Table 3-2). The 0.49 OD increase in HCMV IgG associated with HIV infection also remained after adjustment for age, sex and TB infection status (mean difference 0.47 OD 99% CI 0.37,0.57, $p < 0.001$) (Table 3-2).

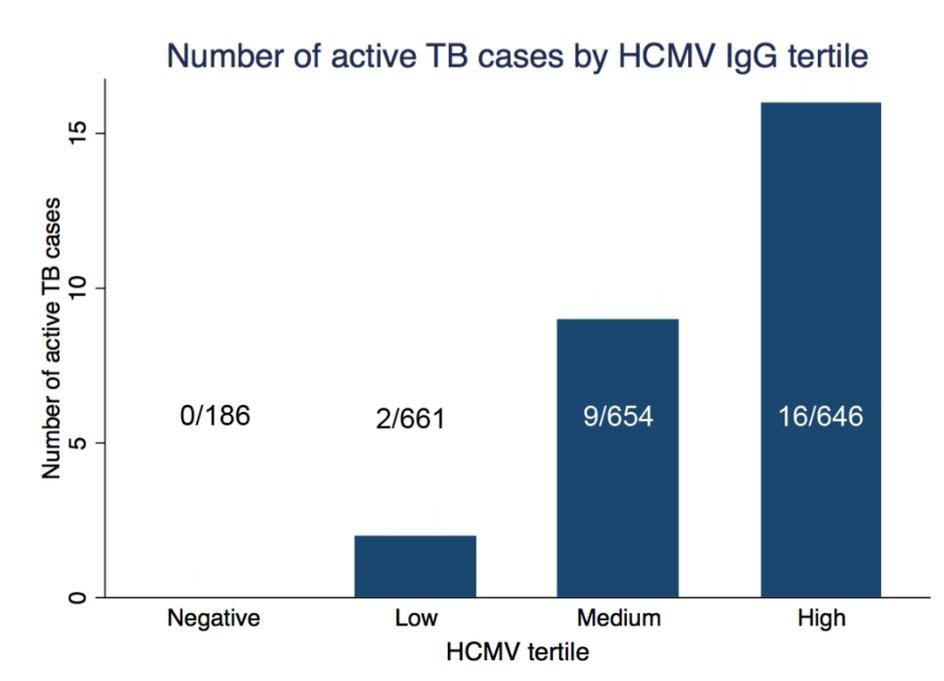
Table 3-2. Unadjusted and fully adjusted mean differences (values obtained using a multivariable model including age, quadratic age, sex, HIV and TB status) in HCMV IgG OD with p value (t test for unadjusted values, regression for adjusted values) and 99% confidence intervals.

Factor (n)	HCMV IgG			Adjusted mean difference	P value	99% CI
	Unadjusted mean difference	P value	99% CI			
Sex						
Male (978)	baseline			baseline		
Female (978)	0.07	<0.001	0.02, 0.12	0.07	<0.001	0.03, 0.11
HIV †						
Negative (1,860)	baseline			baseline		
Positive (96)	0.49	<0.001	0.40, 0.60	0.47	<0.001	0.37, 0.57
TB ‡						
Negative (1,929)	baseline			baseline		
Positive (27)	0.34	<0.001	0.15, 0.53	0.19	0.006	0.01, 0.37

CI - confidence interval, HCMV - human cytomegalovirus, †HIV - human immunodeficiency virus, ‡TB – Active pulmonary Tuberculosis.

Mean HCMV IgG OD levels among the 27 people with sputum-confirmed pulmonary TB was 0.34 OD higher than individuals without active TB. After adjusting for age, sex and HIV infection status this difference is partly explained by those variables but there remains good evidence of that active TB disease is associated with an increased HCMV level of 0.19 OD (99% CI 0.01,0.37, p=0.006) (Table 3-2) which equates to a 7% increase based the range of HCMV OD values (min 0.31, max 2.84 OD). When HCMV seropositivity was divided into low, medium and high responders, the majority of active TB cases had high HCMV IgG levels (16/27, 59%). Thirty three percent were of medium HCMV IgG (9/27), 7% (2/27) had low HCMV, and none were HCMV seronegative (Figure 3-3).

Figure 3-3. Number of active TB cases by HCMV IgG tertile. Numbers above or within bars show number of active TB cases over the total number of individuals within that HCMV tertile (n=27 total active TB cases).



When HIV positive and negative individuals are analysed separately, the effect of active TB disease is shown to be greater in the HIV positive group, but the direction of effect is the same. Among HIV negative people (Table 3-3), having active TB disease is associated with an adjusted

increase of 0.11 OD of HCMV IgG (99% CI -0.10-0.33, p=0.170) whereas among the HIV positive individuals (Table 3-4), having active TB disease is associated with an increased HCMV IgG OD of 0.32 OD (99% CI -0.12,0.77, p=0.063).

Being from a tribe other than the majority Baganda tribe was associated with a 0.14 OD increase in HCMV IgG (99% CI 0.09,0.19, p<0.001). Due to missing data (10/27 active TB cases did not have information on tribe), and the fact that inclusion of tribe into regression analyses did not alter mean difference coefficients, this variable was not included in regression analyses. Although not included, a sensitivity analysis including tribe resulted in a change in coefficient for sex from 0.068 to 0.070 OD, a change in coefficient for HIV from 0.466 to 0.454 and a change in coefficient for TB from 0.193 to 0.184. The coefficient for tribe was 0.11 OD 99% CI 0.06-0.16.

Table 3-3. Unadjusted and fully adjusted mean differences (values obtained using a multivariable model including age, quadratic age, sex and TB status) in HCMV IgG OD with p value (t test for unadjusted values, regression for adjusted values) and 99% confidence intervals for HIV negative individuals only (n=1,860).

Factor (n)	HCMV IgG			Adjusted mean difference	P value	99% CI
	Unadjusted mean difference	P value	99% CI			
Sex						
Male (941)	baseline			baseline		
Female (919)	0.06	<0.001	0.01,0.10	0.06	<0.001	0.02, 0.10
TB ‡						
Negative (1,842)	baseline			baseline		
Positive (18)	0.13	0.142	-0.09-0.35	0.11	0.170	-0.10, 0.33

CI - confidence interval, HCMV - human cytomegalovirus, ‡TB – Active pulmonary Tuberculosis.

Table 3-4. Unadjusted and fully adjusted mean differences (values obtained using a multivariable model including age, quadratic age, sex and TB status) in HCMV IgG OD with p value (t test for unadjusted values, regression for adjusted values) and 99% confidence intervals for HIV positive individuals only (n=96).

Factor (n)	HCMV IgG			Adjusted mean difference	P value	99% CI
	Unadjusted mean difference	P value	99% CI			
Sex						
Male (37)	baseline			baseline		
Female (59)	0.18	0.087	-0.09, 0.45	0.14	0.174	-0.13, 0.42
TB ‡						
Negative (87)	baseline			baseline		
Positive (9)	0.38	0.030	-0.07-0.83	0.32	0.063	-0.12, 0.77

CI - confidence interval, HCMV - human cytomegalovirus, ‡TB – Active pulmonary Tuberculosis.

3.6 Discussion

HCMV is an important pathogen in congenital neurological conditions and in the context of immune suppression. Recent studies have suggested a link between HCMV seropositivity and excess overall mortality within HIC settings [15–19].

Here, we find that 95% of this rural Ugandan population are seropositive for HCMV by age five years (Figure 3-1). This finding corroborates other findings in sub-Saharan African settings where high levels of HCMV seropositivity have been found; 96% in Egypt [20], 97% in Benin [21], 99% in the Gambia [22] and 86% in South Africa [23].

Among the HCMV seropositive population in this study we see high levels of HCMV IgG antibody in young infants under the age of nine months (Figure 3-2). While this may be a true reflection of congenital or primary infection of infants, IgG is the only antibody isotype that can be transferred through the placenta [24], and so these high levels may be driven by the inability of the assay to distinguish between maternal and 'self' IgG. Detection of virus as opposed to antibodies would allow elucidation during the first nine months of life while maternal antibodies persist.

After initial high levels of HCMV IgG in infants under the age of one year, we see a rapid decline whereby maternal antibodies are lost, or primary infection is controlled (but individuals remain seropositive), followed by a gradual increase from the age of 16 years of age up to the highest OD levels seen after 40 years of age. The magnitude of antibody response to virus after maternal antibodies have waned is indicative of intensity of exposure. The finding that females have higher HCMV antibody levels compared to males after adjusting for age, HIV and TB (Table 3-2) was interesting. There is evidence that risk factors throughout life result in a higher risk of HCMV transmission in women compared to men; a study conducted in Uganda using data on breast feeding trends, showed that being a male baby increased the risk of early termination of breastfeeding compared to females [25]. Findings from another Ugandan longitudinal cohort found that age at sexual debut has been consistently lower for women than for men from the 1950's to 1990's [14]. In addition, the infant caregiving role of females results in disproportionate female interaction with infants during the peak shedding at 1-2 years of age [26].

Our results suggest that HIV infection and active TB disease are independently associated with increased levels of HCMV IgG antibodies (Table 3-2). HIV infection is correlated with hypergammaglobulinaemia by way of defective humoral immunity resulting in hyperactivated

naive B cells producing large quantities of IgG [27]. Here we see an association between HIV infection and elevated IgG specific to HCMV, however in the same cohort, we see increased total IgG as well as tetanus toxoid-specific IgG in HIV-infected individuals, an association which remained after adjusting for age and sex (Stockdale *et al*, submitted). Despite this potential confounding of HIV infection upon IgG levels, the increased levels of HCMV IgG in HIV infected individuals is corroborated by epidemiological evidence pointing towards high levels of HIV and HCMV co-infection due to similar routes of transmission [8].

Both TB and HIV are known to increase general inflammation [28,29], an immune environment which is associated with the increased likelihood of HCMV reactivation [30]. Both reactivation and reinfection expose the immune system to HCMV antigens, thereby resulting in elevated levels of HCMV-specific IgG antibodies.

The association of active pulmonary TB with raised HCMV IgG levels was in contrast to findings of a hospital-based study where HCMV-specific T-cell responses in a whole blood assay were found to be lower in TB patients who died when compared to TB patients who survived [31]. In this hospital-based study, HCMV responses were likely a general indicator of 'immune fitness' and therefore predictive of patient survival. By contrast, our study was community based and the severity of TB disease at the time of blood draw would have been low when compared to a hospital-based study.

The association of active pulmonary TB with raised HCMV IgG in our study was consistent with the findings of a correlates of TB risk study in South African infants where risk of active TB disease was increased with an activated T-cell phenotype which was itself correlated with T-cell interferon gamma (IFN- γ) production upon stimulation with HCMV antigens [11]. Neither active case finding, nor routine latent TB infection screening is conducted in the Ugandan GPC and therefore we cannot be certain that 'non-TB' individuals here have not been exposed to TB or indeed that they are not latently infected with *Mycobacterium tuberculosis*. Despite there only being 27 active TB cases within this cohort, there remains strong evidence of an

association between TB and elevated HCMV IgG after adjusting for age and sex; an association which appears to be intensified by co-infection with HIV (Table 3-3 and 3-4).

In view of the 1.5 million deaths per year associated with TB [32], the association of increased HCMV IgG with active TB disease may be an important future research area, especially in areas with large numbers of people living with HIV.

3.7 Limitations

It is important to note that the data from this study give us no information on causality. Longitudinal studies measuring systemic viral loads along with antibody levels to HCMV (and other herpes viruses) in stored sera would be useful to understand progression of both herpes virus and TB disease in this cohort.

3.8 Acknowledgments

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Student	Lisa K Stockdale
Principal Supervisor	Helen Fletcher
Thesis Title	Investigation of immune correlates and impact of human cytomegalovirus and immune activation on tuberculosis disease risk

If the Research Paper has previously been published please complete Section B, if not please move to Section C

SECTION B – Paper already published

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CHAPTER 4 – RESEARCH PAPER

HIV, HCMV and mycobacterial antibody levels: a cross-sectional study in a rural Ugandan cohort

4.1 Chapter Background

This chapter is a research paper, published in Tropical Medicine and International Health. This chapter fulfills aim 2 of this thesis: to measure mycobacteria-specific antibody levels and link these to demographic information and co-infection with HIV and HCMV.

Having characterised the samples for HCMV seropositivity (Chapter 3), this research paper measures mycobacterial antibody levels in the same Ugandan GPC serum samples and uses magnitude of HCMV IgG response as an exposure in regression analysis. The chapter investigates associations of mycobacterial antibody levels with age, sex, along with TB disease and infection with HIV. In this published article, I use the generally accepted assumption that increased HCMV-specific IgG is a result of increased viral exposure however it is important to note that this assumption has not been proven.

4.2 Abstract

Background

A growing evidence base implicates human cytomegalovirus (HCMV) as a risk factor for TB disease. We investigated total IgG and mycobacteria-specific antibodies in a cross-sectional study nested within a rural Ugandan General Population Cohort (GPC), in relation to the magnitude of HCMV IgG response.

Methods

Sera from 2,189 individuals (including 27 sputum positive TB cases) were analysed for antibodies against mycobacteria (Ag85A, PPD, LAM, ESAT6/CFP10) and HCMV, tetanus toxoid (TT) and total IgG.

Findings

Anti-mycobacterial antibodies increased with age until approximately 20 years, when they plateaued. Higher HCMV exposure (measured by IgG) was associated with lower levels of anti-mycobacterial antibodies, but no increase in total IgG. HIV infection was associated with a decrease in anti-mycobacterial antibodies and an increase in total IgG.

Interpretation

The increase in anti-mycobacterial antibodies with age suggests increasing exposure to non-tuberculous mycobacteria (NTM), and to *M.tb* itself. Both HIV infection and high levels of HCMV IgG are associated with decreased levels of mycobacterial antibodies, pointing towards the importance of humoral immune responses in TB disease and highlighting a role of HCMV as a risk factor for TB disease.

This work demonstrates, for the first time, an association between magnitude of HCMV IgG and reduced levels of mycobacterial antibodies.

4.3 Introduction

The role of antibody-mediated immunity in TB has not been fully elucidated (reviewed in [1]). Despite the importance of cell-mediated Th1 responses in TB disease [2,3], attempts to stimulate this arm of the human immune response by vaccine developers have not translated into protection from disease in human efficacy trials [4,5], and there may be useful humoral correlates of disease or protection yet to be identified.

A growing evidence base for the importance of antibodies in protection against other intracellular pathogens such as *Listeria monocytogenes* [6] and *Salmonella spp* [7] has led to renewed interest in humoral responses to TB and the factors which may affect them. Recent work has suggested a potential protective role of antigen 85A (Ag85A)-specific antibodies in reduced TB disease risk in BCG vaccinated South African infants [8,9].

It is known that TB and HIV work synergistically to exacerbate morbidity and mortality in co-infected individuals with respect to both diseases [10]. Epidemiologically, concomitant viral infections other than HIV are known to be associated with poor TB outcomes. In Taiwan Hepatitis C infection was associated with a higher risk of developing active TB disease [11], and in South Africa influenza/TB co-infection was associated with increased mortality [12]. A large TB vaccine trial in South Africa, which investigated correlates of TB disease risk or protection, found an association between CD8 T-cell activation and HCMV response [8] which was linked to increased risk of TB disease and shorter time to diagnosis [13]. The ubiquitous herpes virus HCMV is known to cause immune activation [14], immune senescence [15], and is a significant factor in immune variation [16]. Recently, our group has reported elevated levels of HCMV IgG among TB patients when compared to controls [17]. Despite this, and the findings of some early epidemiologic studies [18,19], data linking HCMV and TB is sparse.

To investigate mycobacteria-specific antibody levels across ages, and to examine potential effects of HCMV co-infection on antibody levels, this study tested 2,187 stored serum samples (of which 27 were active TB cases) from a rural Ugandan cohort for IgG responses to Ag85A, purified protein derivative (PPD), lipoarabinomannan (LAM) and CFP10/ESAT6, along with IgM responses to Ag85A. These antigens were chosen based on availability, evidence of their potential importance in TB disease from the literature (Ag85A [9], LAM [20,21], PPD [22]), and specificity to *M.tb* (CFP10/ESAT6 [23]). Seropositivity to HCMV was measured and existing data on HIV, BCG vaccination status, as well as demographic information was matched and investigated for associations. Responses to tetanus toxoid (TT) and total IgG levels were also investigated as control antibodies.

4.4 Methods

4.4.1 Study area and design

The General Population Cohort (GPC) is a population-based open cohort study in rural south-western Uganda, administered by the Medical Research Council (MRC) UK in collaboration with the Uganda Virus Research Institute (UVRI) [24]. The cohort comprises approximately 20,000 residents (52% aged ≥ 13 years). Data are collected through an annual census, questionnaire and serological survey. Blood specimens are obtained and tested for HIV-1, with the remainder stored at -80°C . The majority of samples used for this study were collected from adults and children in 2011.

Active TB cases were diagnosed through positive sputum smear microscopy after passive case detection and were sampled from a range of GPC rounds between 1999 and 2014. Sera collected as close as possible to the time of TB diagnosis were used for this study.

4.4.2 Sampling

Individuals were selected for inclusion in this cross-sectional study at random after stratification by age and sex. Due to anticipated high levels of HCMV seropositivity, infants under the age of 5 were oversampled. Individuals were only sampled once and siblings and parent-child pairs were not excluded. The total planned sample size was 2,000, plus 10% oversampling. A target of approximately 100 individuals per year of age up to 5 years, 200 individuals aged 6-10, 11-15 and 16-20, 200 in subsequent ten year intervals up to 60 years and 200 people aged 61 years or over. The sex ratio was approximately equal within each age group.

4.4.3 Ethics

Ethical approval for the study was obtained from London School of Hygiene & Tropical Medicine (LSHTM), the UVRI Research and Ethics Committee, and from the Uganda Council for Science and Technology.

4.4.4 Serology: Ag85A, PPD, LAM, CFP10/ESAT6 and TT

Antibody content was measured using ELISA. Experimenters were blind to exposure group for all samples. Testing was conducted at UVRI in Entebbe, Uganda (Ag85A IgG and PPD IgG) and LSHTM, London, UK (CFP10/ESAT6 IgG, Ag85A IgM, LAM IgG and TT IgG). Half volume ELISA plates (Fisher Scientific) were coated overnight at 4°C with sodium carbonate buffer containing 1.5µg/mL recombinant Ag85A protein (Aeras, USA), 1.5µg/mL PPD (Lot 051815KA, Aeras, USA), 0.5µg/mL TT (02/232, NIBSC, UK), 0.5µg/mL LAM (NR-14848, BEI Resources, VA, USA), 0.25µg/mL each CFP10 (NR-49425, BEI Resources, VA, USA) and ESAT6 (NR-14868, BEI Resources, VA, USA). Plates and reagents were brought to room temperature and the plate washed 3 times with PBS, 5% Tween20 (v/v) (PBST). After blocking with PBS 5% milk (w/v) blocking buffer, duplicate 1:100 test serum, high and low PPD responder controls and plate blanks consisting of assay diluent alone with no serum were added in triplicate. Incubation for 2 hours at room temperature, and washing with PBST was followed by a one hour incubation with an appropriately diluted peroxidase-conjugated secondary antibody in PBST-5% (IgG, 1:500 dilution of goat anti-human IgG-HRP (04-10-20, KPL, USA); IgM, 1:10,000 dilution of goat anti-human IgM-HRP (Abcam ab97205, RRID: AB_10695942)). After washing with PBST, 50µL tetramethylbenzidine (TMB, BD BioSciences, USA) was added to each well. The plate was incubated for 15 minutes in the dark before the reaction was stopped by adding 50µL of 2M Sulphuric Acid (Sigma, USA) to each well. Absorbance was measured at 450nm within 30 minutes to obtain optical density (OD), a surrogate marker of antibody titre.

Mean blank values were subtracted from all OD readings, and geometric means of duplicates were used to reduce the effect of skewed distribution. Median levels with interquartile ranges (IQR) are used for graphical representation of antibody levels in figures 4-1 and 4-2.

4.4.5 Total IgG serology

All samples were tested for total IgG content at LSHTM. As above, half volume ELISA plates were coated overnight with mouse anti-human IgG at 0.5ug/mL (Abcam). IgG antibody standards (134.4-8.4 ng/mL) were prepared by diluting purified human IgG (Sigma) in PBST-5% milk and test sera were diluted 8×10^5 in PBST-5% Milk. Duplicate test samples, controls and blanks were incubated for one hour at 37°C, and, after washing, samples incubated with peroxidase-conjugated goat anti-human Fc (Abcam) diluted 1/500 with PBST-5% Milk for one hour at room temperature. Plates were developed with TMB, and sulphuric acid was added to stop the reaction. Plates were read at 450nm and OD measurements converted into g/L by use of the standard curve on each plate.

4.4.6 Serology HCMV

Samples were tested for IgG antibodies against HCMV using a commercial ELISA kit (Novatec Immunodiagnostica GmbH) according to kit instructions (described in [17]).

4.4.7 Sample size estimation

Based on data from a Phase 2b South African infants vaccine study [8], an increase of approximately 0.5 Optical Density units (OD) in Ag85A IgG was observed in the median response in the control vs case infants. These data indicate that a change of 0.5 OD may be biologically meaningful in terms of protection against TB disease. Using power calculations for a type 1 error (alpha) of 0.05, power of 0.80, with an effect size of 0.5 OD, we would require 64 individuals per comparison group.

4.4.8 Statistical analysis

Correlations between each pair of mycobacterial antibody OD levels were conducted using Spearman's rho. Because of the ubiquity of HCMV infection within this population [17], analysis was conducted on the HCMV seropositive population only.

Individuals seropositive for HCMV IgG were further categorized into 3 groups according to tertiles of HCMV antibody concentrations (measured by OD): low, medium and high.

Linear regression was used to determine the association of each antibody response (as continuous dependent variables) with HCMV IgG tertile and HIV infection (as independent variables with HCMV as a categorical variable), adjusting for age and sex. BCG vaccination status and active TB disease were also included in the regression model, however as 89% (24/27) of active TB cases had unknown BCG status, it was not possible to include both variables in the same model. The model including TB was preferred, with the model including BCG run as a comparison to confirm the consistency of the results. BCG vaccination was included in a separate regression analysis using the same model.

Individuals with unknown HIV were excluded from all regression analyses and individuals with unknown BCG vaccination status were excluded from regression analyses including BCG. To account for evidence of non-linearity of antibody OD with age (likelihood ratio test $p < 0.001$ for all antibodies), a quadratic term for age was included in the regression analyses. Numbers included in each of the regression analyses are shown in tables 4-2 and 4-3.

To account for multiple comparisons, 99% confidence intervals (CIs) are reported and a p value of 0.01 is considered to represent strong evidence to reject the null hypothesis. Due to different dynamic ranges of spectrophotometers used in Uganda and the UK, antibody OD measurements (for mycobacterial antibodies only) were rescaled for graphical representation. This was done by dividing each individual's OD value by the maximum OD for that antibody;

hence figure 4-1 shows the percentage of the maximum OD. All analysis was performed using Stata version 14 (Stata Corporation, College Station, TX, USA).

4.5 Results

Sera were sampled from 2,189 individuals. Total IgG levels could not be determined in 15 instances, likely due to protein degradation. Of the remaining 2,174 individuals, 8.6% (186/2,174) of individuals were HCMV seronegative and were excluded from analysis. The HCMV negative population contained no TB cases, 4 HIV positive individuals, 98 (53%) were female and the mean age was 14.6 years (range 3 months-95 years).

The remaining 1,988 individuals were included in further analyses. The mean age was 23.4 years (range 30 days-100 years) and 50% were female (Table 4-1). Ninety-eight percent (1,956/1,988) of individuals had a valid HIV result, and of those, 4.9% (96/1,956) were HIV positive, with the greatest proportion of HIV positive individuals in the 31-50 year age group. Twenty-seven sputum-confirmed TB cases were included in the study. Of these 27 individuals, 63% (17/27) were female and the mean age was 36 years (range 12-59 years). Only three TB cases had BCG vaccination information and all of those were unvaccinated. All 27 were HCMV seropositive, with 59% (16/27) having HCMV IgG levels in the upper tertile.

Table 4-1: Study participant characteristics of evaluable individuals (N=1,988).

Age, mean (range, years)	23.4 (0.08-100.75)
Age of study participants (years):	Number of individuals included in sample:
– <1	– 75
– 1	– 83
– 2	– 82
– 3	– 87
– 4	– 100
– 5	– 106
– 6-10	– 183
– 11-15	– 192
– 16-20	– 204
– 21-30	– 216
– 31-40	– 238
– 41-50	– 166
– 51-60	– 109
– 61 and over	– 147
Total	1,988
Sex, percent female (number/total)	50.0 (993/1,988)
HIV prevalence, percentage (number/total)	4.9 (96/1,956)
BCG vaccinated, percentage (number/total)	70.2 (1,225/1,744)
Active TB cases, percentage (number/total)	1.4 (27/1,988)
Tribe, percentage (number/total)	
Baganda	74.3 (1,400/1,883)
Other *	25.7 (483/1,883)

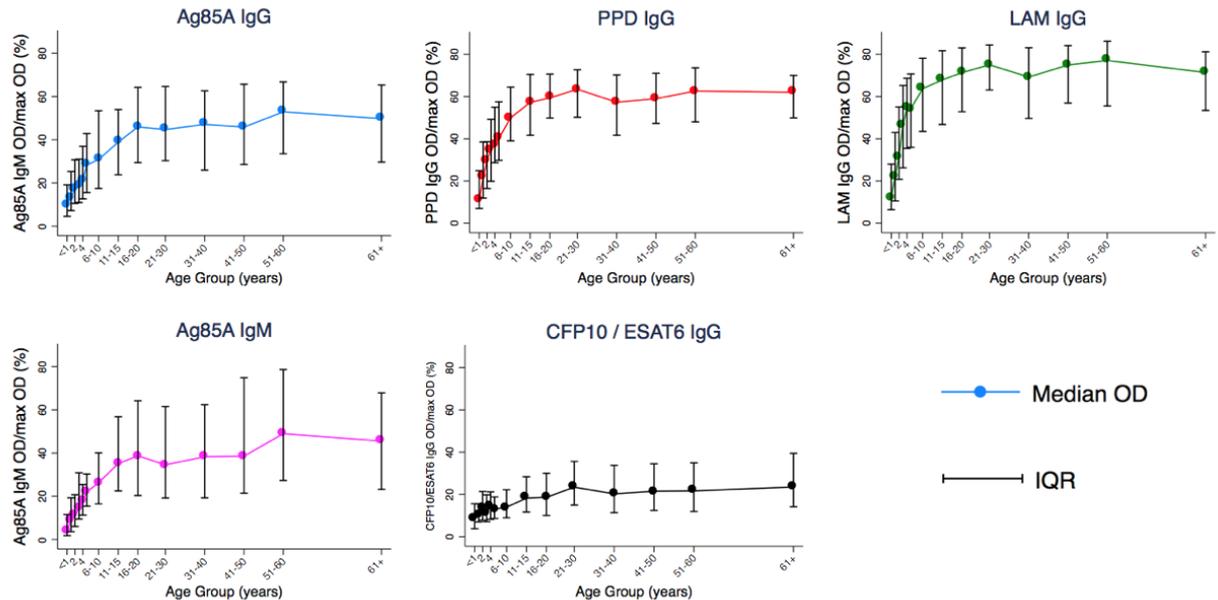
* Other tribes include Rwandese Ugandan, Bakiga, Batooro, Banyankole, Basoga, Bafumbira, Tanzanian, Barundi

4.5.1 Mycobacterial antibody responses increase until late adolescence

IgG against Ag85A, PPD, LAM, CFP10/ESAT6 and IgM against Ag85A all increased from birth to approximately 20 years of age, after which OD values reached a plateau. Figure 4-1 shows

median OD for each of the mycobacterial antibody levels by age group. Initial levels in infants under the age of one year range from a median of 4% of maximum OD (0.12 OD) for Ag85A IgM to 12% of maximum OD (0.35 OD) for LAM IgG. Responses to the *M.tb*-specific ESAT6/CFP10 antigens did not show the same rapid increase from birth as responses to other mycobacterial antibodies. Univariate analysis showed no differences in mean OD levels by sex. Analysis of correlation showed a high degree of co-linearity between all mycobacterial antibodies ($0.33 > \rho < 0.65$, all $p < 0.001$).

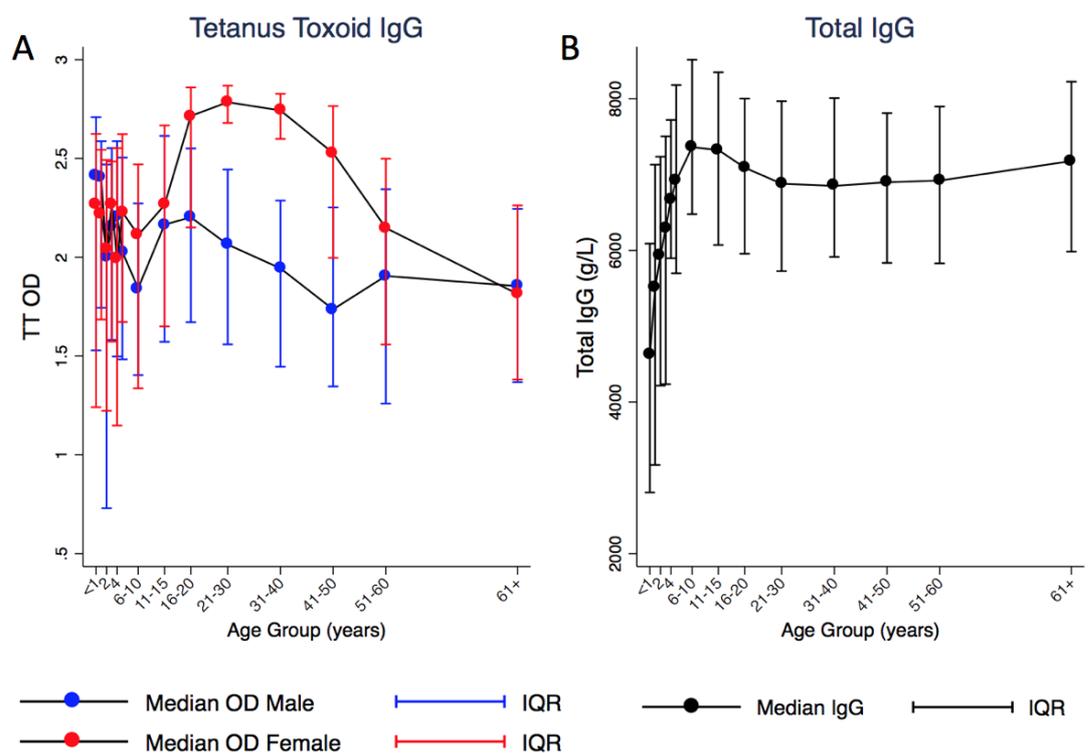
Figure 4-1. Median adjusted mycobacterial antibody OD by age group. Vertical lines show IQR for each median data point. Total n= 1,988. OD values adjusted by dividing OD by the maximum OD response for that antibody. IQR – interquartile range, OD – optical density.



TT and total IgG levels were investigated as control antibodies (figure 4-2). TT vaccination induces high levels of TT IgG and is given at birth and to pregnant females as part of the Uganda immunization schedule [25]. TT IgG showed high levels of antibody from birth and a

clear sex difference with females having higher overall IgG levels ($p < 0.001$, figure 4-2A). This difference is driven by females aged between 16 and 50 years (figure 4-2A). Total IgG levels increase with age from birth (median 46.2g/L in infants less than one year of age) to a plateau after 11 years of age (median 69.4g/L), figure 4-2B. Since a univariate analysis of total IgG by sex indicated no difference, data were not separated by sex for this plot.

Figure 4-2A. Median OD levels for TT IgG by sex and age group. B. Median total IgG (g/L) by age group. Vertical lines show IQR for each median data point. Total n=1,988. Figure 4-2A red lines represent median values for females, blue lines for males. Figure 4-2B shows data for males and females combined. IQR – interquartile range, OD – optical density.



4.5.2 Decreased anti-mycobacterial antibodies with high HCMV IgG and in HIV positive individuals

In a multivariable linear regression model including TB disease status, being HIV positive was associated with a decrease in all mycobacterial antibody levels studied (magnitude ranged from mean decrease of 0.240 OD (99% CI -0.361, -0.119) for IgG against Ag85A), to a mean decrease of 0.446 OD (99% CI -0.640, -0.251) for IgM against Ag85A, all $p < 0.001$, table 4-2). Similarly, being in the upper tertile of HCMV IgG response was associated with a decrease in Ag85A, PPD and LAM IgG levels compared to being in the middle tertile: a mean decrease of 0.067 OD (99% CI -0.122, 0.012) for Ag85A IgG, 0.057 OD (99% CI -0.105, -0.009) for PPD IgG and 0.107 (99% CI -0.194, 0.019) for LAM IgG (all $p < 0.001$, table 4-2). While the direction of change was the same for CFP10/ESAT6 IgG and Ag85A IgM, the mean decrease associated with being in the upper tertile of HCMV IgG response was not sufficiently large to provide good evidence of a difference based upon our significance threshold (table 4-2). The association between mycobacterial antibody levels and magnitude of HCMV IgG response was not linear (Figure 4-3): in comparison to the middle tertile of HCMV IgG response, the lowest tertile was also associated with a decrease in mycobacterial antibody levels. A mean decrease of 0.100 OD (99% CI -0.156, -0.044) $p < 0.001$ was seen for CFP10/ESAT6 IgG and a mean decrease of 0.104 OD (99% CI -0.205, -0.004) $p = 0.01$ for Ag85A IgM. The direction of change was the same for the remaining mycobacterial antibodies (table 4-2).

Figure 4-3. Adjusted mean OD change associated with HCMV tertile comparing to medium HCMV tertile as baseline. P values from multivariable linear regression model including age, quadratic age, sex, HIV and TB. Total n=1,956. Vertical lines show 99% CI for each mean OD change. OD – optical density, CI – confidence interval. (* model includes, age, quadratic age, sex, HIV and TB).

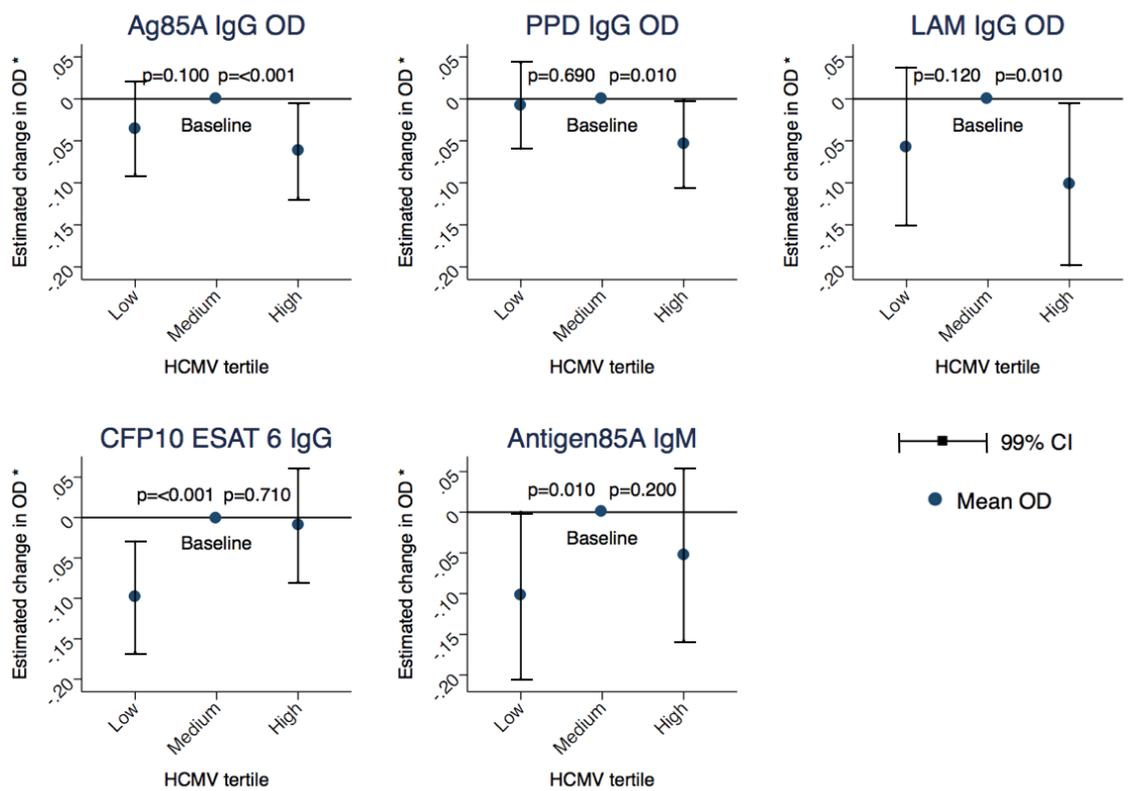


Table 4-2. Fully adjusted mean differences in mycobacterial antibodies (values obtained using a multivariable regression model including age, quadratic age, sex, HIV and TB status, with p values and 99% CI (n=1,956). (CI – confidence interval, OD – optical density)

		Ag85A IgG OD		PPD IgG OD		LAM IgG OD		CFP10 ESAT 6 IgG		Ag85A IgM	
Variable	N (%)	Coeff (99% CI)	P value								
Sex											
Male	978/1956 (50.0%)	baseline									
Female	978/1956 (50.0%)	-0.002 (-0.045, 0.041)	0.9	-0.002 (-0.042, 0.038)	0.91	-0.017 (-0.099, 0.064)	0.58	-0.025 (-0.083, 0.033)	0.27	-0.012 (-0.103, 0.079)	0.73
HCMV Tertiles											
HCMV low	650/1956 (33.2%)	-0.037 (-0.090, 0.016)	0.07	-0.009 (-0.051, 0.033)	0.57	-0.061 (-0.151, 0.030)	0.08	-0.100 (-0.156, -0.044)	<0.001	-0.104 (-0.205, -0.004)	0.01
HCMV medium	650/1956 (33.2%)	baseline									
HCMV high	656/1956 (33.5%)	-0.067 (-0.122, -0.012)	<0.001	-0.057 (-0.105, -0.009)	<0.001	-0.107 (-0.194, -0.019)	<0.001	-0.015 (-0.077, 0.047)	0.54	-0.058 (-0.152, 0.035)	0.11
HIV Status											
Negative	1860/1956 (95.1%)	baseline									
Positive	96/1956 (4.9%)	-0.240 (-0.361, -0.119)	<0.001	-0.310 (-0.422, -0.198)	<0.001	-0.420 (-0.586, -0.253)	<0.001	-0.266 (-0.409, -0.123)	<0.001	-0.446 (-0.640, -0.251)	<0.001
TB Status											
Negative	1929/1956 (98.6%)	baseline									
Positive	27/1956 (1.4%)	-0.101 (-0.322, 0.121)	0.24	-0.035 (-0.217, 0.147)	0.62	0.158 (-0.107, 0.424)	0.13	0.175 (-0.241, 0.590)	0.28	-0.135 (-0.609, 0.340)	0.46

4.5.3 Active TB disease and BCG vaccination: associations with mycobacterial antibody levels

Being a sputum confirmed active TB case was not associated with any differences in any mycobacterial antibody level after adjusting for age, sex, HIV and HCMV level, although given that there were only 27 cases, there was limited statistical power to detect associations (table 4-2). Coefficients of the effect of HIV and HCMV for each outcome measurement were similar for a model including BCG instead of TB, and the magnitude of effect was not altered by adjusting for total IgG. Being BCG vaccinated was associated with a 0.115 OD increase in TT IgG (99% CI 0.004 - 0.227) $p=0.01$.

Table 4-3 shows that HIV positivity was associated with an increase in total levels of IgG (9.110 g/L (99% CI 3.735, 14.485), $p<0.001$). Being female was associated with an increase in TT of 0.272 OD (99% CI 0.188, 0.356, $p<0.001$) but no difference in total IgG levels. While being an active TB case was not associated with any differences in TT IgG, being a TB case was associated with a 5.8 OD decrease (99% CI -13.298, 1.720) in total IgG however this was not significant ($p=0.05$). The magnitude of HCMV IgG response did not affect either total IgG levels or TT IgG response (table 4-3).

Table 4-3. Fully adjusted mean differences in total IgG and TT IgG OD (values obtained using a multivariable regression model including age, quadratic age, sex, HIV and TB status, with p value and 99% CI (n=1,956). (CI – confidence interval, OD – optical density)

		Total IgG (g/L)		TT IgG OD	
Variable	N (%)	Coeff (99% CI)	P value	Coeff (99% CI)	P value
Sex					
Male	978/1956 (50.0%)	baseline		baseline	
Female	978/1956 (50.0%)	-0.326 (-2.350, 1.697)	0.68	0.272 (0.188, 0.356)	<0.001
HCMV Tertiles					
HCMV low	650/1956 (33.2%)	0.318 (-2.240, 2.877)	0.75	-0.090 (-0.187, 0.008)	0.02
HCMV medium	650/1956 (33.2%)	baseline		baseline	
HCMV high	656/1956 (33.5%)	0.915 (-1.508, 3.338)	0.33	0.065 (-0.029, 0.159)	0.08
HIV Status					
Negative	1860/1956 (95.1%)	baseline		baseline	
Positive	96/1956 (4.9%)	9.110 (3.735, 14.485)	<0.001	-0.172 (-0.357, 0.012)	0.02
TB Status					
Negative	1929/1956 (98.6%)	baseline		baseline	
Positive	27/1956 (1.4%)	-5.789 (-13.298, 1.720)	0.05	-0.081 (-0.328, 0.165)	0.4

4.6 Discussion

Previously we have shown that TB patients have increased levels of HCMV IgG [17] and that HCMV positive infants (as measured by HCMV IFN- γ ELISPOT) were at higher risk of progressing to active TB disease [13].

In this study, we found that individuals with the highest levels of HCMV IgG had decreased levels of mycobacterial antibodies, and had no corresponding change in total IgG levels. We found that HIV infected individuals had decreased levels of mycobacterial antibodies, with a concomitant increase in total IgG. These findings suggest that HCMV co-infection, like HIV, may be associated with increased risk of TB disease, and that they both act to specifically deplete mycobacterial antibodies which may be contributing to protective mechanisms against *M.tb* infection.

As has been seen previously [26], humoral responses against RD1 antigens ESAT6 or CFP10 (mycobacterial proteins associated specifically with *M.tb* and not NTM or BCG) did not show an association with active TB cases in this study. Due to a lack of active TB case-finding in the GPC, it is likely that some of the individuals classed as TB negative here were latently infected with *M.tb* based on epidemiologic data on exposure in Uganda [27,28]. The finding that IgG responses to these *M.tb*-specific antigens increase with age, following a similar pattern as other non-*M.tb*-specific mycobacterial antibodies, suggests that while NTM may be driving high responses of acute (IgM) and sustained (IgG) mycobacterial responses, exposure to *M.tb* itself cannot be ignored.

BCG vaccine status allocation in this study was by presence of scar, inspection of immunization card or verbal confirmation by parent of guardian. Despite issues with these methods [29], classification was thought to be robust due to the evidence of association of TT IgG with BCG indicating that people vaccinated for one Extended Programme of Immunization (EPI) schedule

vaccine are more likely to have received other vaccinations upon accessing local health centers.

The magnitude of difference in mycobacterial antibodies seen due to HIV infection was far greater than due to either active TB disease or BCG vaccination. The lower levels of mycobacterial antibodies seen in individuals infected with HIV is not associated with an accompanying decrease in TT IgG or total IgG. The effect seen here points towards HIV infection having a mycobacteria-specific effect as opposed to a more general depression of all antibody levels, thereby confirming evidence seen previously in Italy [30], in this rural Ugandan cohort. Here we see a similar association between the highest levels of HCMV IgG (indicative of highest exposure [31,32]), and decreased levels of some mycobacterial antibodies. Again, this decrease is not accompanied by a decrease in total IgG or TT IgG.

As a human herpes virus, HCMV persists in a variety of cell types in a dormant state and is transmitted through body fluids [33]. Infection is not normally associated with symptomatic disease, however it poses a huge burden on the immune system, with maintenance of up to 30% of both CD4 and CD8 circulating memory T cells being specific to HCMV [34]. The virus is a significant factor in immune variation [16], immunosenescence [15], and causes immune activation [14], including non-specific polyclonal B-cell activation and proliferation [35].

In this study, we see decreased mycobacterial antibody levels at both HCMV IgG extremes among HCMV seropositive individuals. Evidence of HCMV effect on vaccine responses exists, showing both a detrimental impact of HCMV infection [36,37] and a positive, 'adjuvanting', effect of HCMV on vaccine-induced antibody responses in humans [38]. Given reports that cellular responses to heterologous virus challenge in mice may depend on the initial viral dose of HCMV [39,40] the lack of a linear response between HCMV IgG and mycobacterial antibody levels seen in this study may also be indicative of different effects at different HCMV exposure levels. PBMC samples were not available from this cohort for us to determine if magnitude of

HCMV IgG level had a similar impact on the mycobacteria-specific cellular immune response. However, it is likely that cellular immunity is also impacted by HCMV given that HCMV has been shown to affect both humoral and cell mediated immune responses to vaccine antigens [41,42].

Before the widespread use of highly active anti-retrovirals (HAART), HCMV/HIV co-infection saw HCMV as an important cause of severe non-AIDS events, including death, in HIV-infected individuals [43]. High levels of HCMV IgG, caused by repeated reactivation or reinfection events, are associated with higher all-cause mortality [44] and lower CD4 cell count, with accompanying worse response to anti-retrovirals [45]. Despite literature describing HCMV-induced B cell activation [35,46,47], in this study we saw no relationship between HCMV exposure and increased levels of total IgG. We do however see a consistent trend towards decreased mycobacterial antibody response in individuals with very high HCMV IgG responses after adjusting for HIV infection. Considering the risk of developing active TB disease increases over 20 times in individuals with HIV [48], and given the high degree of co-infection of HCMV and HIV due to similar transmission routes [43], it is likely that any added effect of HCMV would be masked. The mycobacterial antibody-specific decrease seen with both HIV infection and, independently, in the highest tertile of HCMV exposure, may be indicative of a functional, protective role of mycobacterial-specific antibodies which are independently decreased by HIV and high levels of exposure to HCMV.

In summary, both HIV infection and high levels of HCMV IgG were both associated with decreases in mycobacterial antibody levels. Given this novel finding of effect of HCMV exposure on mycobacterial antibody levels, investigation of magnitude of HCMV may be important in future TB clinical trials to understand the immune environment, independently of HIV infection status. In addition to quantification of antibody levels, we believe that it will be

important to measure HCMV effect on mycobacteria-specific cellular responses. A more nuanced understanding of the quality, as well as the quantity, of the mycobacterial antibodies elicited, in terms of antibody class, subtype and avidity may help to shed light upon a potential mechanism by which they might confer protection to host cells.

4.7 Limitations

Due to insufficient volumes of sera, we were unable to investigate HCMV viral load in serum samples to ascertain whether active infection at point of blood draw was associated with any of the measurements taken for this study. Despite this, we believe that use of HCMV-specific IgG is a robust a measure of cumulative exposure. As previously mentioned, active TB case finding is not carried out within the GPC and therefore we cannot be sure that some individuals analysed as 'non-TB' are not latently infected. PBMC samples were not available from this cohort for us to determine if HCMV had a similar impact on the mycobacterial cellular immune response.

4.8 Acknowledgements

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SECTION A – Student Details

Student	Lisa K Stockdale
Principal Supervisor	Helen Fletcher
Thesis Title	Investigation of immune correlates and impact of human cytomegalovirus and immune activation on tuberculosis disease risk

If the Research Paper has previously been published please complete Section B, if not please move to Section C

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Where was the work published?	
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Stage of publication	Submitted

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For multi-authored work, give full details of your role in the research included in the paper and in the preparation of the paper. (Attach a further sheet if necessary)	
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Date: 08 September 2018

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Date: 10 September 2018

CHAPTER 5 – RESEARCH PAPER

Case control study of TB in rural Uganda: HCMV infection, but not EBV or HSV, is associated with increased risk of TB disease up to 14 years before diagnosis.

5.1 Chapter Background

This chapter is a research paper written for submission to Lancet Infectious Diseases. This chapter fulfills aim 3 of this thesis: to identify associations between level of HCMV seroreactivity (and other herpes viruses) and markers of inflammation, and aim 4: to investigate links between HCMV infection, measures of antibody quality and TB disease risk.

This research paper measures markers of inflammation in sera from a case control study nested within the cross-sectional cohort from the Ugandan GPC. Measures of chronic herpes virus IgG other than HCMV, as well as antibody avidity of PPD-specific IgG are investigated with respect to risk of TB disease diagnosis.

5.2 Abstract

Background

Recent evidence highlights human cytomegalovirus (HCMV) and immune activation as risk factors for tuberculosis (TB) disease. This study investigates magnitude of HCMV IgG as a risk factor up to 14 years prior to TB diagnosis in a nested case-control study.

Methods

This case-control study used 52 stored serum samples from 26 TB cases stored up to 14 years prior to TB diagnosis from a rural Ugandan cohort. Controls who did not develop overt TB disease were matched on age, sex and HIV. Samples were investigated for herpes virus-specific IgG, serum markers of inflammation and PPD antibody avidity.

Findings

HCMV IgG, but not Epstein Barr (EBV) or Herpes Simplex virus (HSV) IgG was associated with increased risk of active TB disease up to 14 years prior to TB diagnosis. In comparison to individuals with low HCMV IgG, the odds ratio (OR) of people with medium response was 2.2 and for those with high levels was 3.3 ($P_{\text{trend}} = 0.0075$).

Increased PPD-specific IgG avidity associated with decreased risk of TB at early time points prior to TB diagnosis, but with increased risk at point of diagnosis.

Interpretation

These data provide evidence that magnitude of HCMV infection, but not EBV or HSV, is associated with risk of TB disease. In addition, mycobacterial antibody avidity may be a useful measure in the search for immune correlates of TB disease risk.

5.3 Introduction

Tuberculosis (TB) disease is responsible for 1.34 million deaths annually [1]. Epidemiological studies have identified important risk factors for TB: HIV [2], diabetes [3], IFN- γ deficiencies [4], anti-TNF- α therapies [5] and malnutrition [6]. Diagnostics to identify the approximately ten percent of infected individuals that will go on to develop active pulmonary TB disease [7], thereby perpetuating transmission, are still lacking. Recent efforts to discover correlates of risk to direct preventative therapy, or markers of protection to help guide drug and vaccine development have shown promise [8]. Investigation into immune correlates signatures has reconfirmed the importance of IFN- γ and identified a role for IFN $\alpha\beta$ signalling [9], immune activation [10] and antibodies [11], however the mechanisms are not fully understood.

Human cytomegalovirus (HCMV) is a common herpes virus which is transmitted through person-to-person contact. Once infected, HCMV establishes lifelong latency in a variety of cell

types. Infection rarely results in serious side effects in immune competent individuals, however it can cause permanent hearing and neurological damage in neonates and severe non-AIDS events in HIV-infected people [12]. HCMV infection is highly associated with immune variation [13], T cell activation [14], immune senescence, systemic inflammation [15], memory inflation, and increased numbers of B cells [16]. Evidence that accumulated exposure to HCMV correlates with high HCMV IgG titres exists [17,18], but is not proven [19]. Antibody levels are however linked to a range of chronic diseases [20] in addition to increased overall mortality [21]. Previous research from our group and others has observed increased IgG against HCMV in TB cases [22,23], and epidemiologic studies have found a link between HCMV and both TB and non-tuberculous mycobacterial (NTM) disease [23–25]. The mechanisms underlying this relationship have not been elucidated but likely involve sustained chronic inflammation and T cell activation, and may involve alterations in humoral responses.

The objectives of this case control study were to investigate the relationship between extent of HCMV infection, inflammatory markers in serum, anti-mycobacterial antibody avidity as a measure of antibody function, and risk of TB disease in this Ugandan cohort. To determine if the association seen with HCMV is seen in other chronic herpes viruses, samples were also investigated for Epstein Barr (EBV) and herpes simplex viruses (HSV), the latter having good evidence to suggest that antibody levels are directly correlated with viral excretion [26].

5.4 Methods

5.4.1 Sampling

The general population cohort (GPC) is a population-based open cohort based in rural Uganda. The GPC was established in 1989 to examine trends in prevalence and incidence of HIV infection and their determinants [27]. The cohort comprises approximately 20,000 people, half

of whom are aged below 13 years. Data are collected through an annual census and blood samples are stored in a biobank at -80°C, located in Entebbe, Uganda.

TB is diagnosed through passive case identification of symptomatic individuals presenting for care at GPC clinics. Twenty-six individuals from this cohort who were diagnosed with sputum positive active TB disease between 1999 and 2014 were included in this study. From routine samples taken during GPC data collection, we retrieved all available stored serum samples from these 26 TB cases taken up to three months post diagnosis. Between one and four stored serum samples per TB case were identified and retrieved from the biobank of stored samples at time points before, and up to three months after TB diagnosis (total sample number – 52). We categorized the TB case samples into three time point groupings based on number of samples available and anticipated ability to detect differences prior to, and at point of TB diagnosis: between 14 and five years prior to TB diagnosis, between five and one year prior to TB diagnosis, and between one year prior and three months after TB diagnosis (considered point of diagnosis).

For controls, we selected stored serum samples collected in 2011 from people within the GPC who had no record of TB disease. Samples were matched on age, sex and HIV status at time at which sample was taken. Control samples were designated the same time before TB diagnosis grouping as the TB case to which they were matched. Between four and six control individuals were matched per TB case sample (a maximum of one sample per control. Total number of control individuals – 300).

Because of the ubiquity of HCMV infection within this population [22], HCMV seronegative samples were excluded (n=9, all nine were control individuals with a mean age of 37 years (26.9-50.8 years), two of these nine were HIV positive.) The resulting 343 HCMV positive samples, grouped into 52 matched sets, were included in analysis (Table 5-1). Individuals were

further categorized into three groups according to tertile of HCMV antibody concentrations (measured by optical density (OD)): low (n=116), medium (n=119), and high (n=108).

Table 5-1 – Number of TB case samples and matched controls for time periods prior to, and at point of TB diagnosis. The table also shows age, sex and HIV status for the individuals included in this study (for TB case individuals, the mean age is age at sampling closest to diagnosis).

Time before/after TB diagnosis	Non-TB Control samples	TB Case samples	Total
14-5 yrs before	96	17	113
5-1 yr before	90	16	106
1 yr before - 3 months after	105	19	124
Total number of samples	291	52	343
Number of individuals	291	26	317
Mean age (range) yrs	34.45 (2.75-56.5)	36.31(13.08-56.5)	34.48 (2.75-56.5)
Number female (%)	178 (61%)	16 (62%)	193 (61%)
Number HIV positive (%)	87 (30%)	8 (31%)	95 (30%)

The exposure of interest was TB disease as a binary measure. Serum samples were investigated for exposures: IgG against chronic herpes viruses HCMV, EBV and HSV1/2, non-specific inflammatory markers: IFN α 2, IFN γ , IL10, IL12p40, IL12p70, IL1Ra, IL1a, IL1b, IL6, IP10, TNF α , total IgG levels and, antibody avidity of PPD-specific IgG. Known confounders of HCMV IgG level; age, sex and HIV status, are adjusted for by matching controls on these variables.

Testing for HIV was carried out immediately after blood collection in Uganda as previously described by Asiki et al [27].

5.4.2 Ethics

Written consent for the use of clinical records and biological samples for research purposes was obtained from all GPC participants following Uganda National Council of Science and Technology guidelines. Ethical approval for this study was obtained from London School of Hygiene & Tropical Medicine (LSHTM), the UVRI Research and Ethics Committee, and from the Uganda Council for Science and Technology.

5.4.3 Herpes virus-specific IgG

Measurement of HCMV IgG and total IgG was conducted as has been previously described [22]. IgG against Epstein-Barr virus nuclear antigen 1 (EBNA-1) and HSV1 and HSV2 full antigens was measured using commercial kits (Euroimmun, Germany). Testing was conducted as per kit protocol. The resulting measurement (in relative units (RU)) was calculated based on a standard curve from the calibration sera and, based on kit cut-offs.

5.4.4 Luminex

Luminex multiplex cytokine platform was used to determine the concentrations of IFN α 2, IFN γ , IL10, IL12p40, IL12p70, IL1Ra, IL1a, IL1b, IL6, IP10, TNF α in serum samples. Cytokines were tested using Millipore kits (Merck Millipore, USA) according to the manufacturer's instructions. Standard curves were generated from the serial dilutions that were made from the assay controls supplied and matched against the cytokine concentration for quantification. The concentrations of all the analytes in the quality control reagents were found to be within the

ranges as expected. The Bio-Plex manager version 6.1 was used for bead acquisition and analysis of median fluorescence intensity (MFI). MFI was converted to pg/mL.

5.4.5 Total IgG

Half volume ELISA plates (Fisher Scientific) were coated overnight at 4°C with 50µL sodium carbonate buffer containing mouse anti-human IgG at 0.5µg/mL (ab 200699; Abcam). IgG antibody standards (134.4-8.4 ng/mL) were prepared by diluting purified human IgG (0.86 mg/mL; I4506; Sigma) in PBST-5% Milk and test sera were diluted 8×10^5 in PBST-5% Milk. Plates and reagents were brought to room temperature and the plate washed 3 times with PBST. The plate was blocked for one hour at room temperature with 100µL blocking buffer (PBS, 5% Milk (w/v)). Standards and diluted test samples were tested in duplicate and the sealed plate was incubated for one hour at 37°C. The plate was washed five times with PBST and 50ul of peroxidase-conjugated goat anti-human Fc (ab97225; Abcam) diluted 1/500 with PBST-5% Milk was added for one hour at room temperature. Plates were developed using TMB, and sulphuric acid was used to stop the reaction, and read at 450nm. Optical density (OD) measurements converted into g/L by use of the standard curve on each plate.

5.4.6 PPD antibody avidity

Antibody avidity was assessed by ELISA using the chaotropic agent sodium thiocyanate (NaSCN) to dissociate low-affinity IgG binding using a previously described method [28]. An avidity index (AI) was calculated as the molar concentration of NaSCN required to reduce antigen specific IgG binding by 50%.

5.4.7 Statistical analyses

Initial analyses were conducted on all 343 samples in order to investigate differences between individuals who went on to be diagnosed with active TB disease with those who remained TB-free.

Linear regression was used to investigate correlations between continuous serum marker levels, and between serum marker levels and sex, HIV infection status, HCMV tertile, PPD IgG avidity and age.

To investigate associations between serum markers and TB disease, a conditional logistic regression model was used. The model was conditioned on the 52 matched case-control sets and included HCMV tertile as an *a priori* covariate. Due to different measurement ranges, all continuous exposure variable measurements were re-scaled on a 0-10 scale, where zero was the minimum and ten was the maximum observed response for that measurement. Hence reported odds ratios are for a ten percentage point increase in response. Conditional logistic regression analyses of risk of TB disease with increased levels of specific IgG against the other herpes viruses, EBV and HSV were limited by the number of positive individuals. Data on EBV tertile were missing for 19% (64/343) samples and HSV tertile information was missing for 1% (5/343) samples due to IgG levels being below the kit positive cut off. The remaining seropositive samples were divided into 3 groups to form tertiles and sample numbers included in conditional logistic regression analyses are stated in table 5-2.

To determine if the effect of each exposure on TB risk is different in each of the three time periods in which samples were collected, a secondary analysis explored the interaction between the time before diagnosis, and risk of TB disease. For any exposure showing a directional trend in this secondary analysis, a sensitivity analysis was conducted whereby

HCMV tertile was not included in the conditional logistic regression analysis to determine the effect of HCMV.

To account for multiple comparisons, 99% confidence intervals (CIs) are reported and a p value of 0.01 is considered to represent strong evidence to reject the null hypothesis. A robust standard error was employed in conditional regression analysis to account for the fact that some TB cases contributed more than one sample. All analyses were performed using Stata version 14 (Stata Corporation, College Station, TX, USA).

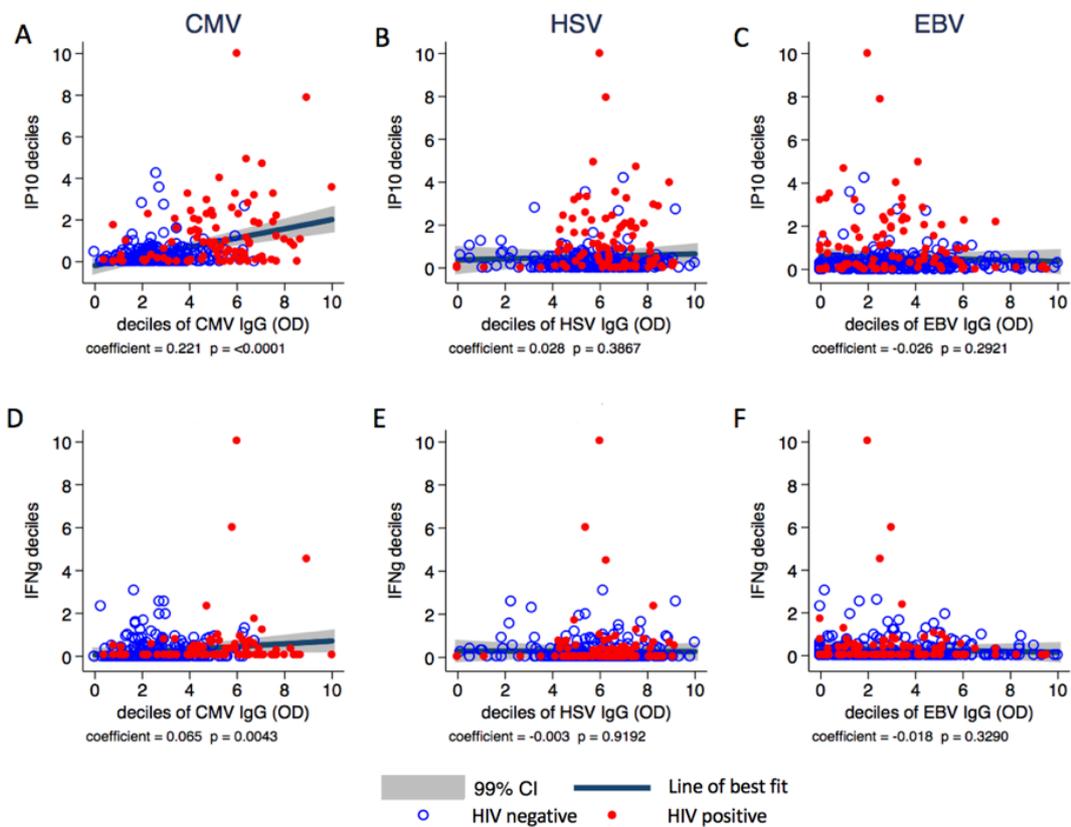
5.5 Results

Of the 317 HCMV seropositive individuals included in analyses, 30% of individuals were HIV positive (8/26 TB cases and 87/291 control samples, (table 5-2). Eighty one percent (279/343) of individuals were EBV IgG positive and 99% (338/343) were HSV IgG positive. HCMV IgG and HSV1/2 IgG were positively correlated (coefficient 0.231, 99% CI 0.082-0.381, $p < 0.0001$). HCMV IgG levels were not associated with EBV IgG (coefficient -0.21, 99% CI -0.135-0.092, $p = 0.631$).

5.5.1 HCMV IgG positively correlates with the serum inflammatory marker CXCL10 (IP10)

Of all the serum levels of inflammatory markers investigated, levels of interferon gamma-induced protein 10 (IP-10) also known as CXCL10, and IFN- γ are positively correlated with HCMV IgG levels (IP10: coefficient 0.221, 99% CI 0.150 – 0.292, $p < 0.0001$, IFN- γ : coefficient 0.065, 99% CI 0.006-0.122, $p = 0.004$). Neither of these are correlated with EBV or HSV1/2 (Figure 5-1).

Figure 5-1 Correlations of IP10 with A) HCMV, B) HSV and C) EBV IgG. Correlations of IFN γ with D) HCMV, E) HSV and F) EBV IgG. Coefficients and P values reported are from an unadjusted linear regression analysis of 343 individuals. Blue hollow markers show HIV negative, and red show HIV positive results.



After adjustment for matching criteria (age, sex, HIV) and TB disease, the regression coefficient between IP10 and HCMV was reduced from 0.221 to 0.185 (99% CI 0.036-0.335, p=0.001) but there remained good evidence of an association. The coefficient for the relationship between IFN- γ and HCMV is largely unchanged (coefficient after adjustment: 0.072, 99% CI -0.063-0.208, p=0.167).

5.5.2 HCMV IgG not associated with increased total IgG

Unadjusted linear regression analysis indicated that increased HCMV IgG was associated with increased total IgG (coefficient 10.80 (99% CI 5.85-15.76) $p < 0.0001$) however, once matching criteria and TB disease were included in the linear regression model, this association was much reduced (coefficient 5.59 (99% CI -0.43-11.61), $p = 0.017$). Neither EBV or HSV1/2 IgG were associated with changes in total IgG.

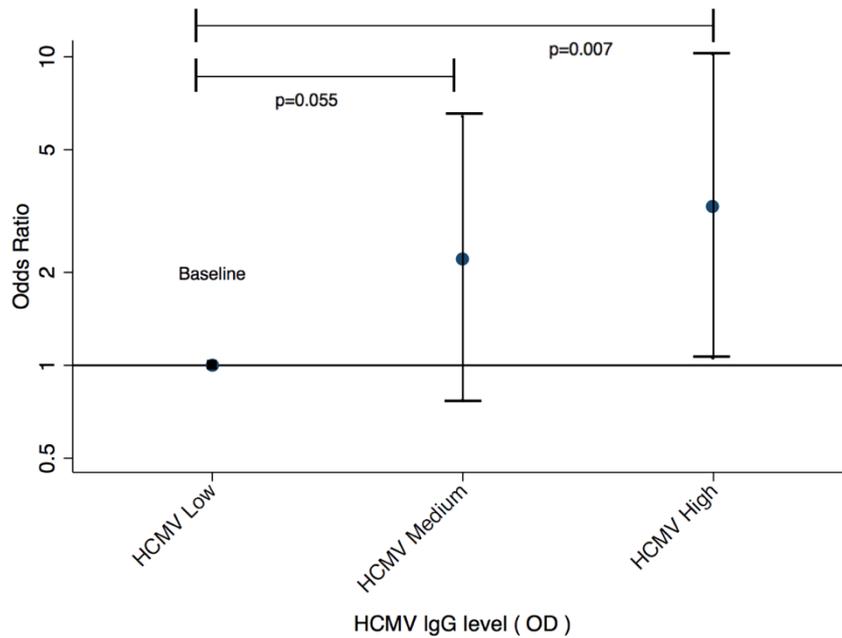
5.5.3 High HCMV IgG, but not HSV or EBV, is associated with increased risk of TB

Initial analyses of association of herpes virus exposure with TB disease risk showed that, irrespective of when the sample was taken prior to TB disease diagnosis, a conditional logistic regression (adjusted for age, sex, HIV status as per study matching) found the odds associated with active TB diagnosis were increased 2.2 times among individuals with an intermediate HCMV IgG compared to low IgG (99% CI 0.764-6.355, $p = 0.055$) (Table 5-2 and Figure 5-2). Having HCMV IgG in the upper tertile of the range was associated with a 3.3 times greater odds of having active TB disease (99% CI 1.052-10.175, $p = 0.007$). The same trend of increased risk with higher IgG was not seen with either EBV or HSV1/2 (Table 5-2).

Table 5-2 – Odds of TB disease by chronic herpes virus IgG level. Medium and high tertiles are compared to the lowest tertile of IgG level for each virus in a conditional logistic regression model including age, sex and HIV status. Odds ratios and 99% confidence intervals are given. The P value is from a likelihood ratio test for trend.

Herpes virus level (n)	Odds Ratio (99% CI)	P _{trend} value
HCMV		
Low (n=116)	1.0	0.0075
Medium (n=119)	2.204 (0.764-6.355)	
High (n=108)	3.272 (1.052-10.175)	
HSV		
Low (n=109)	1.0	0.3363
Medium (n=107)	0.964 (0.387-2.401)	
High (n=110)	0.581 (0.181-1.862)	
EBV		
Low (n= 84)	1.0	0.2271
Medium (n=88)	1.171 (0.423-3.245)	
High (n=85)	0.652 (0.228-1.864)	

Figure 5-2 - Odds of TB disease by HCMV IgG. Results shown are from a conditional logistic regression model which includes age, sex and HIV status. HCMV low is used as the baseline measurement. HCMV low n=116, HCMV medium n=119 and HCMV high n=108. Lines represent 99% CI



5.5.4 Inflammatory markers IL1a and IP10 are associated with increased odds of TB

Again, irrespective of when the sample was taken prior to TB disease, a conditional logistic regression analysis (adjusted for age, sex, HIV status and HCMV tertile) showed that each ten percentage point increase in IL1a within the range seen among this population was associated with a 27% increased odds of being diagnosed with TB up to 14 years prior to diagnosis (OR 1.2695, 99% CI 1.0148, 1.5880, p=0.006, table 5-3). Each ten percentage point increase in IP10 was associated with a 233% increased odds of being diagnosed with active TB disease (OR 3.3 99% CI 1.2-9.1, p=0.002, table 5-3). Each ten percentage point increase in total IgG was

associated with 29% reduced odds of being diagnosed with active TB disease (OR 0.71, 99% CI 0.51-0.98 p=0.007, table 5-3).

Table 5-3 - Odds of TB disease in a univariate conditional logistic regression model including age, sex, HIV status and HCMV tertile. The Odds ratio reported here is per 10 percentage point increase in that marker. OD – optical density.

	Mean (range)	Odds Ratio (OR)	99% CI	P value
Inflammatory markers (unit of measurement)				
IFN α 2 (pg/mL)	16.98 (0-502.9)	1.299	0.823, 2.050	0.140
IFN γ (pg/mL)	3.80 (0-134.8)	1.340	0.936, 1.918	0.036
IL10 (pg/mL)	19.50 (0-2711.4)	2.710	0.547, 13.427	0.109
IL12p40 (pg/mL)	4.79 (0-440.8)	1.020	0.617, 1.685	0.919
IL12p70 (pg/mL)	0.87 (0-34.2)	0.979	0.696, 1.376	0.869
IL1R α (pg/mL)	11.70 (0-904.3)	1.296	0.840, 1.999	0.124
IL1 α (pg/mL)	51.67 (0-805.7)	1.270	1.015, 1.588	0.006
IL1 β (pg/mL)	49.2 (0-746.6)	1.020	0.749, 1.388	0.870
IL6 (pg/mL)	109.61 (0-4700)	1.126	0.871, 1.457	0.234
IP10 (pg/mL)	298.9 (0-5533.6)	3.327	1.211, 9.140	0.002
TNF α (pg/mL)	81.5 (0-768.7)	1.018	0.8130, 1.274	0.834
Other serum markers				
Total IgG (g/L)	68.7 (19.52-117.3)	0.711	0.514, 0.983	0.007
PPD IgG avidity index (OD)	3.0 (1.86-5.0)	1.004	0.817, 1.234	0.962

5.5.5 PPD antibody avidity is associated with changing risk of TB disease over time

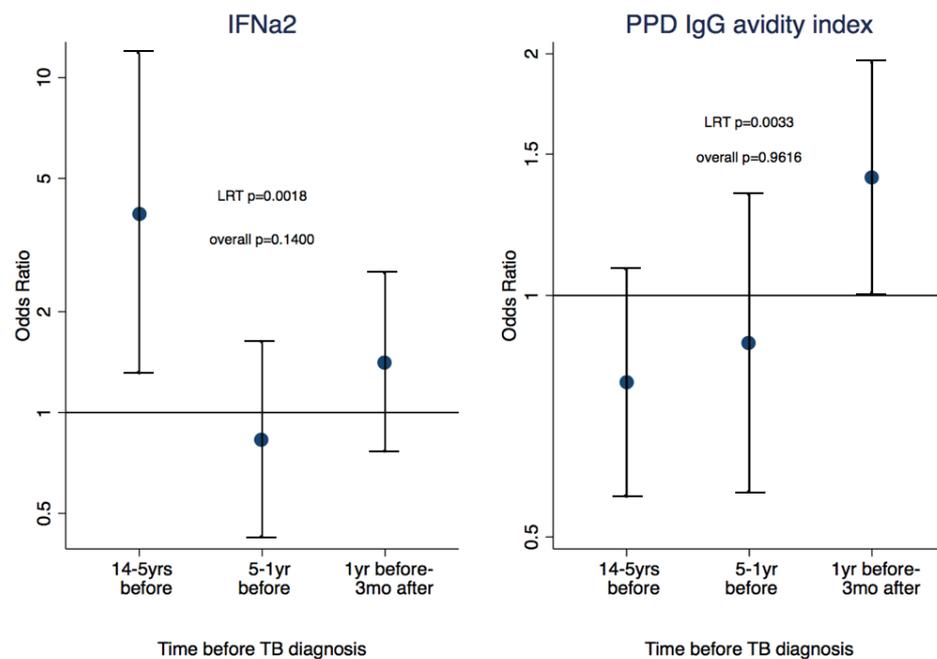
A likelihood ratio test (LRT) provided good evidence of an interaction between time before diagnosis and odds of TB disease risk for serum IFN α 2 level and PPD IgG avidity (Table 5-4).

There was no evidence of an interaction with time for any other exposure variable.

Table 5-4. Investigating changes in serum IFN α 2 levels and PPD IgG avidity at different time points prior to TB disease. The p value reported is from the LRT. The OR and 99% CI reported are from a conditional logistic regression model (conditioned on matching criteria (age, sex, HIV status) including HCMV tertile and an interaction with the time point at which the sample was taken. The odds ratio reported is per ten percentage point increase in either IFN α 2 or PPD IgG avidity at the time point tested.

Variable	Odds Ratio	99% CI	P value (from LRT)
IFN α 2			
14-5 yrs before diagnosis	3.920	1.305,11.77	0.0018
5-1 yr before diagnosis	0.831	0.425,1.626	
1 yr before-3 mo after diagnosis	1.407	0.763,2.597	
PPD avidity index			
14-5 yrs before diagnosis	0.778	0.561, 1.078	0.0033
5-1 yr before diagnosis	0.872	0.569, 1.335	
1 yr before-3 mo after diagnosis	1.399	1.006, 1.947	

Figure 5-3 – Graphical representation of odds ratios associated with univariate conditional logistic regression (model including matching criteria (age, sex, HIV status) and HCMV tertile) for IFN α 2 and PPD IgG avidity index according to the time before TB diagnosis that serum samples were taken. The odds ratios and 99% CIs are representative of a ten percentage point increase in the respective exposure.



For IFN α 2, there was no directional trend in the run up to TB diagnosis: between 14 and five years before diagnosis, a ten percent increase in serum levels of IFN α 2 is associated with increased risk of TB disease; between five and one year before diagnosis, the same increase was associated with decreased risk; and at point of diagnosis, with increased risk (figure 5-3 and table 5-4).

For PPD IgG avidity, between 14 and five years prior to TB diagnosis, a ten percent increase in avidity of PPD-specific antibodies is associated with a 22% decreased risk of TB diagnosis (OR

0.778, 99% CI 0.561, 1.078). Closer to point of diagnosis, the same increase in avidity is associated with a 12.8% decrease in risk (OR 0.872, 99% CI 0.569, 1.335). At point of TB diagnosis, each ten percentage point increase in PPD IgG avidity is associated with a 40% increased risk of TB diagnosis (OR 1.399, 99% CI 1.006, 1.947). These results are not affected by the adjustment for HCMV IgG tertile. In a sensitivity analysis where HCMV tertile was not included in the conditional logistic regression analysis, the overall P value from the LRT investigating the interaction of time with PPD IgG avidity changed from 0.0033 to 0.0074. The ORs for each time point are altered by less than four percent.

5.6 Discussion

It is estimated that approximately one quarter of the world's population is infected with TB [29]. Identification of the 55.5 million people who are at high risk of TB disease [29] will be crucial to halt the epidemic. There remain many gaps in our knowledge as to the reasons for some individuals' ability to control infection with *M.tb* while others develop active disease. Immunological risk factors include IFN- γ deficiencies and decreases in T cell count: HIV infection being one of most important associations. Recent evidence implicates both immune activation, inflammation, and the human herpes virus HCMV as risk factors for TB disease.

In this study, we used a case control design containing longitudinal samples taken before, and at point of TB diagnosis to investigate the relationship between HCMV, inflammatory markers, and risk of TB disease. We found that magnitude of HCMV infection, as measured by IgG, was associated, in a dose-dependent manner, with risk of TB disease up to 14 years prior to diagnosis. Well established confounders of HCMV and TB including age, sex and HIV were accounted for in the study design. The same association with TB risk is not seen with EBV or HSV despite evidence of co-linearity between HCMV and HSV seen here and elsewhere [30], and evidence of co-prevalence of these three chronic herpes viruses [31].

Our findings are consistent with a study in infants where a HCMV-specific T-cell response (measured by IFN- γ enzyme-linked immunospot (ELISpot)) was associated with increased risk of, and decreased time to, infection with TB [32]. HCMV infection is normally evaluated by serology, and high HCMV IgG titres are linked to accumulated HCMV burden [20,21]. It is not known whether the association between increased HCMV IgG, increased overall mortality [21], and chronic diseases [20] is also observed with HCMV-specific T cell responses. One study showed general concordance between antibody and cellular immune responses against HCMV, however the group found no association between HCMV IgG titre and quantitative HCMV-specific ELISpot responses, and over a third of HCMV IgG seronegatives demonstrated a positive HCMV ELISpot response [33].

Here, we see that being among the highest HCMV IgG group, risk of TB disease is increased 3.3 times. HCMV seropositivity is ubiquitous in this population [22], and the assay used to quantify HCMV is semi-quantitative. The grouping into tertiles of HCMV-specific IgG is based upon the ranges seen in this population and may not be generalizable. It will be important to understand if the range seen here is similar to other populations and what specific cut-offs of HCMV IgG are associated with increased risk of TB.

Circulating levels of most cytokines measured were below the threshold of assay sensitivity, however IP10 serum levels were generally high in this population and were further elevated in individuals with high HCMV IgG. This positive correlation between IP10 and HCMV was not seen with EBV or HSV. Along with other systemic immune activation markers such as serum C-reactive protein and IFN- γ , elevated levels of IP10 have been associated with post-transplantation morbidity in HCMV discordant recipients [15]. It is known that an interferon-inducible gene profile, including IP10, is associated with active TB disease [9] however it is not

understood if it is TB itself, latent TB infection, or an underlying cause, such as viral co-infection, which is driving this profile [34]. IP10 is positively correlated with HCMV IgG levels however IL1 α is not. Interestingly here we see that IP10 and IL1 α are themselves associated with increased odds of TB disease after adjustment for HCMV IgG tertile, indicating that while risk of TB due to HCMV may be due to HCMV-mediated effects, a HCMV-independent inflammatory profile may also be linked to risk of disease.

The only exposure that showed evidence of an interaction between time before TB diagnosis and clear directionality of response was PPD antibody avidity. Investigation of PPD IgG avidity at different time points prior to TB disease diagnosis showed a trend suggestive of highly avid antibodies specific to mycobacterial antigens having a positive, protective effect up to 14 years prior to TB diagnosis. Measures of mycobacteria-specific antibody avidity prior to TB diagnosis have, to our knowledge, not been studied before. Work conducted by Perley *et al* found that active TB disease in humans was associated with a decrease in avidity of antibodies able to bind directly to the surface of live bacteria [35]. This finding, and our work, point towards the importance of qualitative analysis of antibodies; antibody avidity being one measure of quality that has been shown to be critical for protection induced by the meningococcal B vaccine [36].

In summary, this work shows, in this rural Ugandan population, that odds of TB disease are increased to 2.2 times among individuals with intermediate HCMV IgG, and 3.3 times among individuals with high levels of HCMV IgG. This finding is independent of HIV infection, age and sex.

An inflammatory environment, here characterized by high IP10 and IL1 α , is associated with increased risk of TB disease in this rural Ugandan cohort. Further work is needed to understand if the results found here are generalizable to other populations. Identification of robust correlates of TB disease risk up to 14 years prior to diagnosis would be transformative

to the TB field. We believe that the data presented here warrant further research into the effect of magnitude of HCMV IgG levels, the possible protective effect of mycobacterial antibodies, and investigation of the immune environment in future TB clinical trials.

The implications of this work for TB control efforts are that, given the excess TB disease risk associated with HCMV, HCMV infection should be given the same importance as other known TB risk factors such as HIV and diabetes. Development of an HCMV vaccine is already underway [37], however target groups mainly consist of women of child bearing age to protect neonates from HCMV-associated neurological disorders. The data presented here show that the need for HCMV control measures may be greater than initially predicted.

5.7 Limitations

Although the capacity exists within the GPC to collect PBMC, cells were not available for the current study. The normal study design of a case-control cohort dictates that controls be matched to cases at point of diagnosis. Using stored serum samples, and only having one serum sample per non-TB control, we were unable to match in this way retrospectively. This may introduce a potential source of bias for this study. Control samples were matched to case samples on age, sex and HIV status when the sample was taken. In order to mitigate this bias, one individual who was diagnosed with active TB disease changed HIV status during the sampling time and so was excluded. The passive case-finding within the GPC likely underestimates the true burden of disease. The control individuals included in this study were not investigated for TB infection. More stringent inclusion criteria for controls may have resulted in larger differences between groups.

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Chapter 6 – UNPUBLISHED RESULTS

Use of Mycobacterial growth inhibition assay (MGIA) to investigate ability of serum to inhibit mycobacterial growth

6.1 Chapter Background

Results chapters 3 and 4 have characterised HCMV IgG and mycobacterial antibodies respectively. Chapter 4 described associations between mycobacterial antibody levels and HCMV, HIV, BCG vaccination status and TB disease. Chapter 5 investigated inflammatory markers and PPD-specific antibody avidity and linked those with HCMV, EBV and HSV, and risk of TB disease at different time points before TB diagnosis.

Based on the findings that HCMV IgG levels were higher in TB cases than in individuals who were not diagnosed with active TB disease (chapter 3); that high levels of HCMV IgG were associated with decreased levels of the mycobacterial antibodies Ag85a, PPD and LAM (chapter 4); and that highly avid PPD-specific antibodies were associated with a directional trend to decreased odds of TB many years prior to TB diagnosis, the functional activity of serum to inhibit mycobacterial growth is investigated in this final results chapter. This chapter is not prepared for publication.

6.2 Introduction

The *ex-vivo* mycobacterial growth inhibition assay (MGIA) is a functional assay that aims to provide a summative assessment of immune mechanisms and their interactions within a biological sample [1]. Initially supported by the WHO, the MGIA was developed to overcome the lack of defined immune correlates of vaccine efficacy in human studies. Use of a standardized, validated stock of BCG specifically prepared for use in the MGIA, instead of

variable quality stocks of *M.tb* helped to increase assay reproducibility, and avoid the use of biosafety level 3 facilities. A good correlation was seen between BCG and *M.tb* in comparative studies [2].

MGIA, using quantification based on the BACTEC mycobacteria growth indicator tube (MGIT) automated system, has been shown to discriminate between individuals by their functional ability to control bacillary growth following vaccination [3]. As opposed to a single measure of host immune response such as the IGRA, this assay provides an unbiased assessment of mycobacterial control as a measure of anti-mycobacterial immunity. Normally the MGIA is used to measure cell-mediated immunity induced by developmental vaccines in animal models [4] and humans [1] using serum of bovine origin to provide growth factors in cell culture.

To determine if serum from TB cases or individuals with particularly high or low levels of anti-mycobacterial, and HCMV, antibodies was having any effect on the ability of human peripheral blood mononucleocytes (PBMC) to inhibit mycobacterial growth, the MGIA was utilised here.

6.3 Methods

PBMC were isolated from one UK-born, BCG-unvaccinated volunteer from LSHTM (see Chapter 2.8 for methodology). These cells were tested for IFN- γ by ELISPOT (see Chapter 2.9 for details), and used in a BCG MGIA where cells were combined with heat inactivated sera from individuals within the GPC (see Chapter 2.10 for full methods of MGIA). Briefly, 3.5×10^6 PBMC were combined with 20% serum from one of 37 individuals from the GPC chosen based on either being a confirmed TB case or levels of Ag85A, LAM or HCMV IgG within the highest or lowest 20% of the range seen in the samples included in the nested case control (see Chapter 2.2.4 for sampling). All serum donors were randomly selected from HIV negative individuals over the age of 15 years. BCG vaccination status was not specified in the sample selection.

Controls consisting of commercial FBS and human AB serum and IgG-depleted AB serum were included in duplicate. Positive controls consisting of a mixture of PHA and PMA were included in duplicate.

A final input of BCG of 90 CFU in 300 μ L was added to the PBMC and serum and left to incubate for 4 days. After this, cells were washed, lysed and added to a BACTEC MGIT tube. The system is based on fluorescence quenching by oxygen. As oxygen levels within the tube are depleted by bacterial growth, fluorescence is released and measured hourly by the BACTEC machine which gives a 'time to positivity' (TTP) readout. This readout is compared to a 'direct-to-MGIT' where BCG is inoculated directly into the tube. The readout of TTP was converted to log CFU of BCG in the sample based on a standard curve. A total of 37 test samples were included in singlicate, and controls in duplicate.

6.3.1 Statistics

Data were analysed using GraphPad Prism 7 and Stata version 14 (Stata Corporation, College Station, TX, USA). An age and sex adjusted linear regression model was used to investigate associations between log CFU and BCG vaccination and log CFU and sex. Associations between log CFU and levels of mycobacterial antibodies, herpes virus IgG, inflammatory markers as well as total IgG and PPD-specific IgG avidity were investigated using linear regression model adjusting for age and sex.

The non-parametric Mann-Whitney test was used to compare median log CFU between two groups (for example TB cases vs controls, BCG vaccinated vs BCG unvaccinated, and males vs females).

6.4 Results

6.4.1 ELISPOT results

The UK-born LSHTM-based volunteer who was used as the source of PBMC was tested for IFN- γ release following T-cell stimulation with various mycobacterial antigens. The positive control of PMA and PHA was unreadable due to blackout (too many spots to count), confirming that the cells could produce IFN- γ upon stimulation by a mitogen. Unstimulated cells provided a background reading of 1.75 SFU. After background subtraction, the individual was negative for reactivity to ESAT6/CFP10 and LAM antigens (2.75 and 0.75 SFU respectively). The individual had positive responses to PPD and Ag85B antigens with mean SFU measurements of 51.75 and 12.5 respectively.

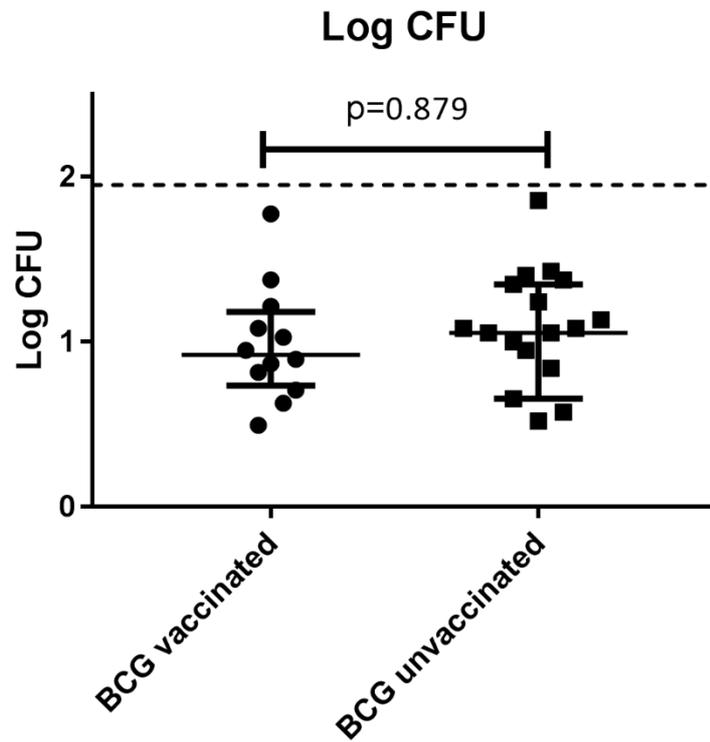
6.4.2 MGIA Results

Associations between log CFU and BCG vaccination were investigated in an age and sex adjusted linear regression model. Of the 37 individuals selected for inclusion in the MGIA experiment, 51% (19/37) were historically BCG vaccinated, 35% (13/37) were not vaccinated and 14% (5/37) had missing data on BCG vaccination status. No association was found between BCG vaccination status and ability to control BCG growth in the MGIA (coefficient= 0.03, 99% CI -0.47- 0.53, p=0.879) (Figure 6-1).

Similarly, no associations were seen between males and females in an age-adjusted model (coefficient= -0.046, 99% CI -0.55- 0.45, p=0.805).

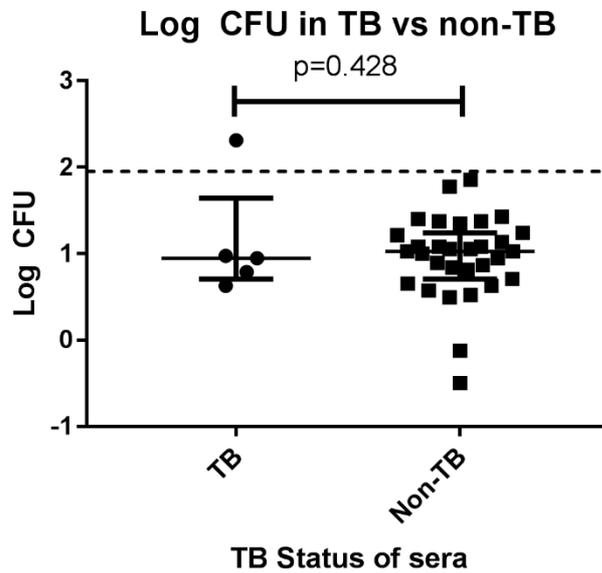
Figure 6-1: Log CFU of serum from BCG vaccinated (n=19) and unvaccinated (n=13) individuals.

Dashed line represents input dose of 90 CFU BCG.



TB cases were compared to non-TB in Figure 6-2 below. The spread of data is large for both groups and there was no evidence of a difference between the 2 groups (coefficient=0.20, 99% CI -0.48 - 0.88, p=0.428).

Figure 6-2: Log CFU of serum from TB cases (n=5) and serum from individuals without active TB disease (n=32). Dashed line represents input dose of 90 CFU BCG.



Results from a minimally adjusted linear regression model (including age and sex) of log CFU growth and deciles of exposures including mycobacterial antibody levels, herpes virus IgG, inflammatory markers as well as total IgG and PPD-specific IgG avidity are shown in Table 6-1 below. None of the exposures show any significant association with the ability of the serum to inhibit growth at the 0.01 significance level.

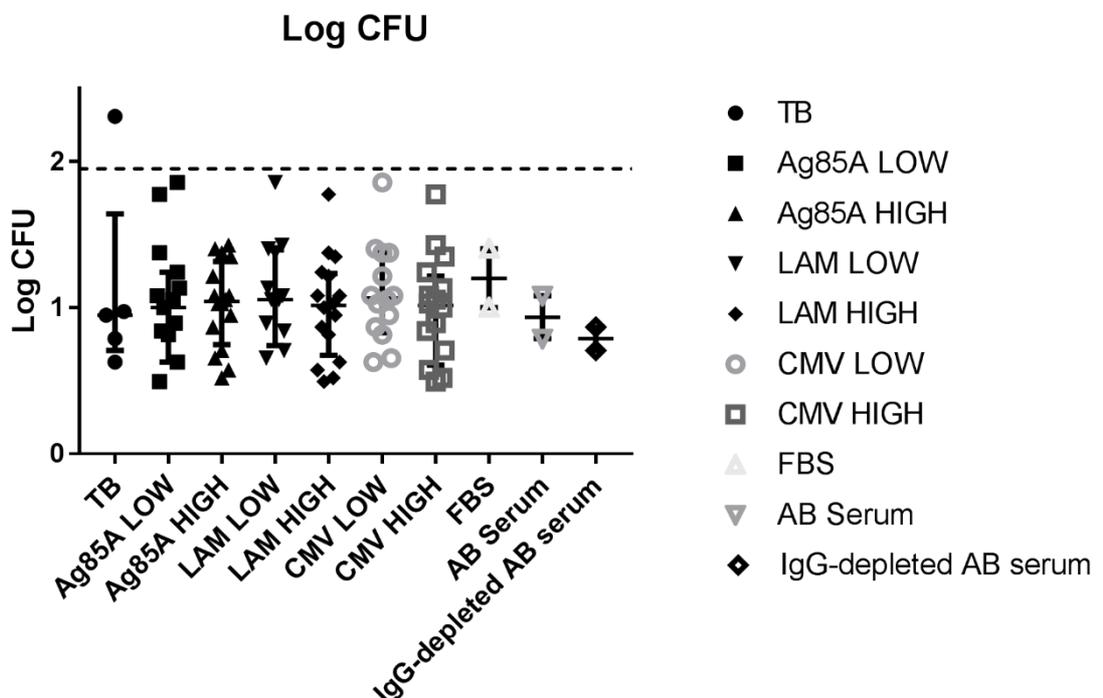
Table 6-1. Associations between mycobacterial growth inhibition and levels of exposures using a linear regression model adjusted for age and sex. The coefficients reported here are per 10 percentage point increase in that marker. OD – optical density.

Variable	mean (range)	coefficient	99% CI	P value
Mycobacterial Antibody levels				
Ag85A IgG (OD)	0.91 (0.23, 1.69)	0.0121	-0.0623, 0.0866	0.6585
PPD IgG (OD)	1.18 (0.50, 1.70)	-0.0058	-0.1097, 0.0982	0.8802
LAM IgG (OD)	1.94 (0.35, 2.73)	0.0233	-0.0656, 0.1123	0.4776
CFP10/ESAT6 IgG (OD)	0.69 (0.07, 1.98)	-0.0386	-0.1361, 0.0589	0.2853
Ag85A IgM (OD)	1.46 (0.27, 2.78)	-0.0123	-0.0879, 0.0632	0.6569
Herpes virus IgG levels				
HCMV IgG (OD)	1.07 (0.20, 1.75)	-0.0419	-0.1526, 0.0688	0.3077
EBV IgG (OD)	76.27 (0, 197.34)	0.0197	-0.0706, 0.1100	0.5553
HSV IgG (OD)	145.52 (32.92, 245.11)	-0.0526	-0.1570, 0.0518	0.1772

Inflammatory markers				
IFNa2 (pg/mL)	12.20 (0, 149.48)	-0.0375	-0.1568, 0.0819	0.3963
IFNg (pg/mL)	3.69 (0, 30.83)	0.0437	-0.0531, 0.1405	0.2254
IL10 (pg/mL)	7.78 (0, 47.81)	0.0146	-0.0643, 0.0934	0.6167
IL12p40 (pg/mL)	3.87 (0, 62.12)	-0.0092	-0.1155, 0.0972	0.8152
IL12p70 (pg/mL)	0.42 (0, 6.31)	-0.0194	-0.1225, 0.0837	0.6092
IL1Ra (pg/mL)	4.88 (0, 75.87)	-0.0136	-0.1316, 0.1043	0.7536
IL1a (pg/mL)	38.33 (0, 267.51)	0.0388	-0.0494, 0.1269	0.2372
IL1b (pg/mL)	5.89 (0, 140.71)	0.0115	-0.1333, 0.1563	0.8286
IL6 (pg/mL)	95.21 (0, 1846.34)	0.0076	-0.1059, 0.1211	0.8551
IP10 (pg/mL)	165.97 (0, 1951.46)	0.0956	-0.0274, 0.2187	0.0411
TNFa (pg/mL)	12.99 (0, 183.27)	0.0404	-0.0845, 0.1653	0.3822
Other serum markers				
Total IgG (g/L)	69.57 (46.84, 104.68)	-0.0089	-0.1082, 0.0903	0.8067
PPD avidity index (OD)	3.02 (2.07, 4.68)	0.0436	-0.0435, 0.1307	0.1799

Figure 6-3 shows all groups plotted by the antibody levels for which the samples were chosen. Sample selection ensured that sera selected for inclusion were within the upper or lower 20% of measurements from samples included in the larger nested case-control study. Of the 32 samples chosen for inclusion, 16 of those had Ag85A IgG levels within the top 20%, and 16 had Ag85A IgG levels within the lower 20%. Similarly, 16 were LAM IgG high, 16 LAM IgG low, 16 were HCMV IgG high and 16 were HCMV IgG low. Figure 6-2 shows that there were no differences between ability to inhibit BCG growth based on the Ag85A IgG, LAM IgG or HCMV IgG level within the serum.

Figure 6-3: Log CFU for all antibody level groups. Dashed line represents input dose of 90 CFU BCG.



Control samples consisting of FBS, AB serum and IgG-depleted AB serum were measured in duplicate. A high degree of variability was seen between these duplicates, with a coefficient of variation of 24%, 22%, and 14% respectively (Figure 6-3).

6.5 Discussion

Serum samples chosen for this assay were selected based upon their Ag85A, LAM or HCMV IgG content. Due to time constraints of this project, sample selection was carried out prior to the analysis of data for Chapter 5. In retrospect, given the link between PPD antibody avidity and risk of TB disease (Chapter 5), it may have been better to select samples based on that parameter instead.

In order to minimise variation, PBMC from only one donor was used in this experiment. Only 50mL of blood can be taken at any one time from a volunteer and so the number of cells available for use in this assay was limited. This therefore limited the number of comparison groups in addition to the number of replicates and controls included.

The MGIA has been optimised over several years to determine the best mycobacterial input (BCG vs *M.tb*), the multiplicity of infection (how many bacteria per host cell), as well as parameters such as use of whole blood compared to PBMC, and antibiotic content in the culture medium [5].

Prior to optimisation, the MGIA suffered from high intra-assay variability and could not detect vaccine-induced differences [6]. However, more recent studies have shown much lower levels of variability and higher consistency. In a study which investigated mycobacterial growth inhibition in the presence of pre- and post- primary or secondary BCG vaccination with THP-1 cells, it was found that lower BCG growth was seen in post-vaccination sera compared to pre-vaccination [7]. It was also found that both enhanced growth inhibition and enhanced phagocytosis was associated with IgG against arabinomannan [8].

The ELISPOT results showed that the PBMC, despite originating from a non-BCG vaccinated individual, may have some reactivity to environmental mycobacteria sharing PPD and Ag85B antigens. While it would have been ideal to test several donors, and use PBMC from someone with no T-cell responses to any mycobacterial antigens, that was not possible given the time and resource limitations of this project. The PPD and Ag85B positivity seen here is indicative of exposure to NTM which may be interacting with the mycobacteria in the MGIA and masking any effect due to serum levels.

Here, we saw no associations between historic BCG vaccination and ability to control BCG growth *ex-vivo*, nor a link between active TB disease and growth. We also did not see any associations between serum antibody levels and growth. However, It is likely that the assay system used here was not sufficiently optimised for the assessment of a heterologous serum response on donor PBMC. The use of donor PBMC limited the number of input cells per well which likely increased variability in the assay and restricted the number of individuals available for each group which meant the study was underpowered to observe differences between groups. Also, the pre-existing immune response to mycobacterial antigens discussed above could have interfered with our ability to observe differences.

The lack of difference between groups could also be due to other constituents of serum. The sera used here were only characterised for levels of 3 antibodies (Ag85A, LAM and HCMV IgG) but many others could be having an effect. Despite the samples having been heat inactivated to remove any effect of complement, there are likely to be other differences such as serum cytokine and chemokine levels not measured in the luminex assay.

In conclusion, the MGIA assay was not able to show that sera from individuals with differing levels of certain antibodies was associated with any difference in ability to inhibit mycobacterial growth in this situation. It is likely that the assay was not sufficiently optimised and underpowered to see such difference due to the reason discussed above. In addition, serum samples used in this experiment were not sufficiently characterised and likely had high intrinsic heterogeneity, therefore masking any effect on mycobacterial growth. Evidence discussed previously in this thesis suggests that serum constituents can affect the ability of bacteria to enter cells and replicate within them. Therefore, given more time and resource, including development of more robust procedures with more stringent criteria for reactivity of donor PBMC, (and the use of a cell line to ensure large numbers of cells) the MGIA may well be able to differentiate between sera, if any real difference actually exists.

6.6 References

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CHAPTER 7: OVERALL DISCUSSION AND CONCLUSIONS

7.1 Summary and significance of findings

Taken together, the data presented in this thesis add to the growing evidence that HCMV infection and TB disease are linked. In Chapter 3, I show that in this Ugandan cohort, HCMV seropositivity is ubiquitous. Higher HCMV IgG levels are linked to female sex, HIV infection, and active TB disease. In Chapter 4, I show that mycobacterial antibody levels increase with age, a finding indicative of cumulative exposure to NTM and *M.tb* over a lifetime. HIV infection, one of the most important risk factors in TB disease, is associated with dramatic decreases in all mycobacterial antibody levels investigated. The same pattern is seen with decreased mycobacterial antibodies in the highest tertile of HCMV IgG. Both HIV and HCMV show a much greater magnitude of effect on mycobacterial antibody levels compared to either active TB disease, or historic BCG vaccination.

Having elucidated that increased HCMV IgG is linked to active TB disease in this cohort, and that high levels of HCMV are associated with decreased mycobacterial antibodies, I then wanted to investigate a link between HCMV and risk of TB disease in a nested case control study. In Chapter 5, I show that up to 14 years prior to TB disease diagnosis, higher levels of HCMV IgG are associated with increased risk of TB disease in a dose-dependent manner. The same associations are not seen with other chronic herpes viruses EBV or HSV.

Also in Chapter 5, I showed that PPD antibody avidity was associated with a directional trend to increased odds of TB disease as time to TB diagnosis decreased. Having highly avid PPD antibodies associated with a more protective phenotype at time points distal to diagnosis, which switched to being associated with risk at point of TB diagnosis. To test the hypothesis that HCMV may be affecting the quality of the antibody response, I investigated the interaction between HCMV IgG

level and PPD antibody avidity. The data did not support the hypothesis that HCMV IgG level was affecting PPD antibody avidity.

While more work needs to be done to tease apart the mechanism by which HCMV may be affecting risk of TB disease, the work in this thesis clearly shows that HCMV is associated with risk of TB in this cohort. Spatial and temporal similarities between the two pathogens underscore the logic of a hypothetical interaction. Both TB and HCMV infect the same cell types in the same organ; both infect macrophages and can establish chronic latency in these cells in the lung [1–3], and NK cells play a critical role in the innate immune response against both pathogens [4,5]. As we saw in Chapter 3, HCMV infection is almost ubiquitous by age 5 in this Ugandan cohort [6] and this is representative of other LMICs [7]. The early exposure of the immune system to HCMV, a pathogen associated with immune activation and inflammation [8], may predispose HCMV-infected individuals to infection with TB and ultimately, to increased susceptibility to development of active TB disease. Data is scarce on prevalence of LTBI however one study of 290 participants in Kampala, Uganda (75 % of whom were aged between 15–34 years) showed that overall prevalence of LTBI was 49% with older age being a significant risk factor [9]. While we cannot infer causality by the age at which infection occurs, in Uganda it seems as though infection with HCMV occurs earlier than infection with TB.

In chapter 1 showed that extent of HCMV IgG, but not EBV or HSV, was positively correlated with the inflammatory marker IP10 (CXCL-10), and risk of TB disease also increased with increasing levels of IP-10, independently of HCMV IgG levels. IP10 is a potent inflammatory chemokine induced by IFN- γ . Specifically interacting with CXCR3 receptor, a surface molecule expressed on activated T and B and NK cells, IP10 induces chemotaxis of those activated cells to the site of secretion [10]. Latent HCMV infection has been shown to induce host cells to secrete pro-inflammatory cytokines, including IP-10, into the extracellular environment [11,12]. In the absence

of viral exposure (indicative of latent HCMV infection [13]), taken here to be driving HCMV antibody responses [14], HCMV induces IP10 production by host cells, thereby insinuating additional risk associated with HCMV infection. Abnormal levels of IP10 have been observed in a variety of diseases including infectious diseases and cancer [15]. Therefore, while the potential risk of TB disease attributable to HCMV infection could well be a combined effect of both HCMV exposure (measured by IgG) in addition to HCMV-induced IP10 secretion, this hypothesis requires further research.

It is important to highlight that the assumption used throughout this thesis is that HCMV IgG accurately reflects HCMV viral exposure. There remains conflicting evidence for this assumption [14,16]. There may be alternative mechanisms by which certain groups of individuals mount stronger humoral responses to HCMV infection. Unfortunately, we were unable to study possible genetic associations as only serum was available for the individuals analysed in this PhD. It is known that genetic variation in the human NKG2 receptor which determines ability of host cells to lyse HCMV-infected target cells [17] impacts susceptibility, but in addition, sex hormones have a large effect on immunity, and specifically inflammation [18]. Further work is needed to understand if there exists a genetic predisposition to high CMV titres, however females generally have higher antibody levels and B cell numbers than men [19].

If, as is inferred from temporality of infection with the two pathogens, HCMV is acting to increase risk of TB disease, mechanisms by which HCMV may be doing this could be either TB-specific or more general. Results from the nested case-control do not show any evidence of a HCMV-specific alteration to mycobacterial antibody avidity (Chapter 5). The lack of PBMC in this project meant we were not able to investigate any effect of HCMV on mycobacteria-specific T-cells. Given the association between HCMV and T-cell activation [20], it is possible that increased HCMV exposure,

could be or driving an apoptotic T cell phenotype [21], or, like HIV infection, could be specifically depleting TB-specific T cells [22]. Given the importance of NK cells in both pathogens [4,5], another possible mechanism by which HCMV might increase risk of TB could be the preferential trafficking of NK cells with anti-viral potential as opposed to anti-mycobacterial. Again, further work would be needed to test this hypothesis. Alternative mechanisms by which HCMV infection could be causing increased risk of TB disease might not be TB-specific. HCMV encodes many viral proteins including UL111A; a homologue to the Th2 cytokine IL-10 [23]. IL-10 is well known for regulating effector T cell immune responses, including inhibiting production of the protective pro-inflammatory cytokine, IFN- γ [24]. Mouse models have highlighted the importance of IL-10 in TB infection and progression [25,26]. In humans, this has been linked to IL-10-dependent blocking of phagosome maturation [27]; an essential mechanism by which the phagosome and lysosome fuse, creating the phagolysosome capable of microbial degradation [28]. Innate lymphoid cell (ILC) subsets participate in early immune responses against foreign antigens by production of various cytokines [29,30]. Like Th1 cells, ILC1 cells produce IFN- γ upon interaction with pathogens [31]. Lung-resident ILCs (including natural killer (NK) cells) are among the first cell subsets to be exposed to inhaled *M.tb* bacilli and are thought to be crucial to the outcome of infection [32]. ILCs in the gut are regulated by ILC regulatory cells (ILCregs) which act via IL10 to control inflammation [33]. Because HCMV infection occurs very early in this population [6], it is possible that virally-encoded IL-10 could be interfering with innate immune control of initial TB infection, leading to a more TB-permissive phenotype at a young age.

Much as HIV infection status would always be taken into consideration when conducting a TB clinical trial, the data presented in this thesis support the idea that not only HCMV infection status, but extent of HCMV infection, should also be taken into consideration. This finding may well

extend to other pathogens other than TB which are affected by an inflammatory environment. These data may also add urgency to the HCMV vaccine effort.

The role of antibodies in TB is still unclear, however the finding here that co-infections associated with increased risk (HIV and HCMV) act to decrease mycobacterial antibody levels, and that mycobacterial antibody avidity changes in the run up to TB disease may help to direct further mechanistic investigation of their importance.

Both HCMV and TB have been associated with lower socio-economic status. Large studies from the USA [34] and the UK [35] found HCMV seropositivity of 50% in age-adjusted analysis, and of 70% in individuals over 65 years of age respectively. Both studies found a clear association between risk of HCMV infection and lower socio-economic status. To our knowledge, no work has investigated correlation between socio-economic status and HCMV as a continuous variable in areas of high HCMV seropositivity. An association between risk of TB and low socio-economic status in India was largely attributable to lower education level and poverty [36,37]. Among foreign-born individuals in the USA, evidence showed that TB risk and socio-economic status were only weakly associated [38]. In this rural Ugandan cohort, not only is HCMV infection ubiquitous, but differences in socio-economic status are much less pronounced than in the USA and UK. Since all GPC participants are provided with access to healthcare, socio-economic status is calculated based on variables such as ownership of household items, wall type and roof quality [39]. Approximately 13% of the residents attain education beyond primary level, and the majority of people are employed in subsistence farming [40]. There are no tarmacked roads within the GPC and buildings are mostly semi-permanent structures made from locally available materials [41]. Despite the smaller scale differences in socio-economic status within the GPC in comparison to some high income countries, early studies of the HIV epidemic in Uganda showed that the heads of the poorest households were more likely to be HIV positive [42].

Socio-economic status was not investigated as a covariate in this project, and it, along with maternal education, may be a useful potential confounder to include in future analyses. In addition, determination of viral load and shedding by PCR of urine and or saliva would be very informative to understand what exactly high HCMV IgG levels are actually measuring. Longitudinal urine, saliva and blood samples would allow a greater understanding of whether reinfection and reactivation events are driving HCMV IgG. One interesting point to be kept in mind however, is that, given the long-term presence of the MRC research base providing healthcare, treatment and general health education, measurement of socio-economic status within the GOC may not be generalizable to other areas of Uganda.

As previously discussed, research into HCMV vaccine development [43] mainly focusses on women of child bearing age to protect neonates from HCMV-associated neurological disorders. The data presented in this thesis show that the need for HCMV control measures may be greater than initially predicted given the excess TB disease risk associated with HCMV.

7.2 Study strengths and limitations

TB research usually relies on small sample sizes due to the paucity of funding and the high cost of clinical trials. The large sample sizes used here allowed greater granularity in the investigation of mycobacterial antibody levels by age. The investigation of vaccines in the 2011 GPC round allowed the inclusion of BCG vaccine status in analysis.

Using an established cohort such as the GPC, while a phenomenal source of data and biological material, does also have its limitations. Individuals are not routinely investigated for TB or LTBI. Therefore, some 'non-TB individuals' are likely to have LTBI that has not been identified. With

more stringent diagnosis, I may have been able to see differences in mycobacterial antibody levels, or data from the MGIA would have been clearer. In a similar vein, BCG vaccination status, although corroborated by TT IgG levels indicative of access of healthcare and EPI schedule vaccines, is not entirely robust.

The GPC has recently started collecting PBMC however these were not available for the years studied for this PhD. With stored PBMC, we would be able to test for LTBI, as well as investigate mycobacteria-specific cellular changes associated with extent of HCMV infection.

As mentioned previously, TB is not actively diagnosed and so I was limited by the total number of individuals diagnosed with active TB disease with accessible samples. Because of this, I was under-powered to look at trends in antibody and cytokine levels at various points leading up to TB diagnosis in the nested case control study.

Despite meticulous planning and scheduling of experiments to minimise amounts of serum used, limitations of financial resource as well as physical band width resulted in prioritisation of assays and depletion of some samples.

7.3 Opportunities for future work

The points raised through this research beg the question as to whether the same associations would be evident in geographic areas where HCMV infection is not as ubiquitous but where TB disease is an important cause of mortality and morbidity. Examples being homeless people in high income countries or among incarcerated populations.

Despite not having access to matched samples from an area with lower HCMV prevalence, in this study we do see that overall total IgG levels are much higher than reference values. Among the 2,174 individuals included in the cross-sectional analyses, the mean total IgG levels are normally

distributed around a mean of 6650 mg/dL with a range of 162 – 13118 mg/dL. The reference values for total IgG levels range between 500-1330mg/dL (mean 886.5 mg/dL) for Iranian adolescents [44] and 407–2170 mg/dL (mean 1118 mg/dL) for adults from Spain [45], and 251-1349 mg/dL (mean for adults 994mg/dL) for US individuals [46,47]. As highlighted in work conducted for the first manuscript of this PhD thesis [6], there seems to be a paucity of information on reference values of widely-used measurements used for clinical diagnosis in sub-Saharan African settings [48]. Although different techniques were used to study total IgG levels in the studies referenced above, the values found here in this study are over ten-fold higher than those of higher income countries. This work suggests that fundamental immune parameters should have reference values which are specific to the population of interest. The cross-sectional cohort used here would be well suited to investigate ranges of commonly-used reference values.

Further work to continue the investigation into the importance of antibodies in TB may be fruitful. IgG subclasses exhibit functional differences in Fc receptor binding and complement activation; IgG1 and IgG3 are the most effective subclasses at complement activation whereas IgG2 can only weakly, and IgG4 cannot activate complement at all. IgG1 is the subclass most effective at complement-dependent cell lysis. In the example of HIV, elite controllers of viremia were linked to a higher IgG1/IgG3:IgG2/IgG4 profile which was independent of overall magnitude of antibody levels and individual effector functions [49]. Following recent work which identified differential glycosylation of the Fc portion of mycobacterial antibodies as being linked to active TB disease compared to LTBI, the samples from the nested case control study would be well placed to investigate qualitative changes such as this.

Additionally, further investigation into the individuals seronegative for HCMV may be interesting. In an area with almost ubiquitous infection, it seems strange that some individuals are either not

exposed, or do not mount detectable antibody responses to HCMV antigens. This phenomenon is not unique to this cohort, seronegative individuals have been seen in studies across Africa [7]. It would be interesting to understand if these results are due to inadequate diagnostic tests or if these individuals really are seronegative. If the results are real over serial samples, are there any genetic associations or clinical advantages associated with protection from HCMV infection?

7.4 References

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Our Ref: GC/127/15/06/512
Your Ref:

04th June 2015

Dr. Robert Newton,

RE: UVRI REC review of protocol titled **“A sub-study of antibodies against tuberculosis nested within two Ugandan cohorts”**

Thank you for submitting the response to the queries addressed to you by the UVRI REC.

This is to inform you that your responses dated 02nd June 2015 were reviewed and met the requirements of the UVRI REC.

UVRI REC annual approval has been given for you to conduct your research up to 04th June 2016. Annual progress report and request for extension should be submitted to UVRI REC prior to the expiry date, to allow timely review.

The reviewed and approved documents included;

1. UVRI REC Application form
2. Study Proposal
3. Applicant's CVs

You can now continue with your study after registration with the Uganda National Council for Science and Technology (UNCST).

Note: UVRI REC requires you to submit a copy of the UNCST approval letter for the above study before commencement.

Yours sincerely,

Mr. Tom Lutalo
Chair, UVRI REC
C.C The Director-UVRI,
Secretary, UVRI REC



Observational / Interventions Research Ethics Committee

LSHTM

8 July 2015

Dear

Study Title: A study to investigate the BCG-specific antibody response within two Ugandan cohorts

LSHTM Ethics Ref: 10000

Thank you for responding to the Observational Committee's request for further information on the above research and submitting revised documentation.

The further information has been considered on behalf of the Committee by the Chair.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation as revised, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Covering Letter	LSHTM Ethics committee response letter 6JULY2015	06/07/2015	V1

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: <http://leo.lshtm.ac.uk>

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

**Professor John DH Porter
Chair**

ethics@lshtm.ac.uk
<http://www.lshtm.ac.uk/ethics/>



Observational / Interventions Research Ethics Committee

Miss Lisa Stockdale
LSHTM

26 April 2016

Dear Lisa ,

Study Title: Investigation of immune correlates in Tuberculosis disease risk

LSHTM ethics ref: 10643

Thank you for your application for the above research, which has now been considered by the <Observational/Interventions> Committee.

Confirmation of ethical opinion

On behalf of the Committee, I am pleased to confirm a favourable ethical opinion for the above research on the basis described in the application form, protocol and supporting documentation, subject to the conditions specified below.

Conditions of the favourable opinion

Approval is dependent on local ethical approval having been received, where relevant.

Approved documents

The final list of documents reviewed and approved by the Committee is as follows:

Document Type	File Name	Date	Version
Investigator CV	CV HFletcher Nov2015a	14/03/2016	v1
Investigator CV	CVLisa StockdaleFEB2016	14/03/2016	v1
Protocol / Proposal	Study Protocol 10485 14MAR2016	17/03/2016	v2
Information Sheet	Information Sheet and Consent Form 14MAR2016	17/03/2016	v2

After ethical review

The Chief Investigator (CI) or delegate is responsible for informing the ethics committee of any subsequent changes to the application. These must be submitted to the Committee for review using an Amendment form. Amendments must not be initiated before receipt of written favourable opinion from the committee.

The CI or delegate is also required to notify the ethics committee of any protocol violations and/or Suspected Unexpected Serious Adverse Reactions (SUSARs) which occur during the project by submitting a Serious Adverse Event form.

At the end of the study, the CI or delegate must notify the committee using an End of Study form.

All aforementioned forms are available on the ethics online applications website and can only be submitted to the committee via the website at: <http://leo.lshtm.ac.uk>

Additional information is available at: www.lshtm.ac.uk/ethics

Yours sincerely,

Professor John DH Porter
Chair

ethics@lshtm.ac.uk
<http://www.lshtm.ac.uk/ethics/>

ELISA PLATE WORKSHEET – ELISAs MRC UGANDA JULY-AUGUST 2015

ANALYST:	
DATE:	
ANTIGEN:	
PLATE NUMBERS:	
DILUTION FACTOR:	
SAMPLE INFO	

Stock concentrations: PPD – 0.5mg/mL, 85A – 0.82mg/mL, Goat anti-human – 0.1mg/mL (after reconstitution)

REAGENTS

Reagent	Final concentration	Stock concentration	Batch number	Volume Required (Solution and Stock)
Capture Antigen				
Positive control				
Detection Antibody				
Coating buffer	N/A	N/A		N/A
Blocking buffer	N/A	N/A		N/A
Other reagents				

PLATE INCUBATION TIMES

INCUBATION STEP	START DATE/TIME	STOP DATE/TIME
Plate Coating (O/N)		
Blocking (1hr)		
Sample and Controls (2hrs)		
Detection Antibody (1hr)		
Substrate Development (15-25mins)		
Stop Reaction		N/A

Plate Reading		N/A
---------------	--	-----

ELISA PLATE MAP

42 samples per plate (1 dilution in duplicate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1 1:100	Sample 2 1:100	Sample 3 1:100	Sample 4 1:100	Sample 5 1:100	Sample 6 1:100	Sample 7 1:100	Sample 8 1:100	Sample 9 1:100	Sample 10 1:100	Sample 11 1:100	Sample 12 1:100
B	Sample 1 1:100	Sample 2 1:100	Sample 3 1:100	Sample 4 1:100	Sample 5 1:100	Sample 6 1:100	Sample 7 1:100	Sample 8 1:100	Sample 9 1:100	Sample 10 1:100	Sample 11 1:100	Sample 12 1:100
C	Sample 13 1:100	Sample 14 1:100	Sample 15 1:100	Sample 16 1:100	Sample 17 1:100	Sample 18 1:100	Sample 19 1:100	Sample 20 1:100	Sample 21 1:100	Sample 22 1:100	Sample 23 1:100	Sample 24 1:100
D	Sample 13 1:100	Sample 14 1:100	Sample 15 1:100	Sample 16 1:100	Sample 17 1:100	Sample 18 1:100	Sample 19 1:100	Sample 20 1:100	Sample 21 1:100	Sample 22 1:100	Sample 23 1:100	Sample 24 1:100
E	Sample 25 1:100	Sample 26 1:100	Sample 27 1:100	Sample 28 1:100	Sample 29 1:100	Sample 30 1:100	Sample 31 1:100	Sample 32 1:100	Sample 33 1:100	Sample 34 1:100	Sample 35 1:100	Sample 36 1:100
F	Sample 25 1:100	Sample 26 1:100	Sample 27 1:100	Sample 28 1:100	Sample 29 1:100	Sample 30 1:100	Sample 31 1:100	Sample 32 1:100	Sample 33 1:100	Sample 34 1:100	Sample 35 1:100	Sample 36 1:100
G	Sample 37 1:100	Sample 37 1:100	Sample 38 1:100	Sample 38 1:100	Sample 39 1:100	Sample 39 1:100	Sample 40 1:100	Sample 40 1:100	Sample 41 1:100	Sample 41 1:100	Sample 42 1:100	Sample 42 1:100
H	+ve Control Serum 1:100	+ve Control Serum 1:100	+ve Control Serum 1:100	-ve Control Serum 1:100	-ve Control Serum 1:100	-ve Control Serum 1:100	Assay Diluent	Assay Diluent	Assay Diluent	Plate Blank	Plate Blank	Plate Blank

REAGENT WORKSHEET – ELISAs MRC UGANDA JULY-AUGUST 2015

ANALYST:	
DATE:	

10X PBS

BATCH NUMBER:	
DATE MADE:	
EXP:	N/A

TO A 1L BEAKER, ADD:

REAGENT	AMOUNT	LOT NUMBER	TICK WHEN ADDED
NaCl	80g		
KCl	2g		
Na ₂ HPO ₄	14.4g		
KH ₂ PO ₄	2.4g		
DEIONIZED WATER	800mL		
ADD STIR BAR AND LEAVE SPINNING UNTIL DISSOLVED CHECK pH – should be 7.4 If pH is higher than desired, use Hydrochloric acid, if lower, use sodium hydroxide Raise volume to 1L with deionized water			

COATING BUFFER

BATCH NUMBER:	
DATE MADE:	
EXP:	

REAGENT	AMOUNT	LOT NUMBER	TICK WHEN ADDED
NaHCO ₃	8.4g		
Na ₂ CO ₃	3.56g		
DEIONIZED WATER	1L		
ADD STIR BAR AND LEAVE SPINNING UNTIL DISSOLVED CHECK pH – should be 9.5 If pH is higher than desired, use Hydrochloric acid, if lower, use sodium hydroxide Raise volume to 1L with deionized water			

WASH BUFFER – MAKE 5L FOR 20 PLATES

1XPBS w/0.05% T20 (v/v)

BATCH NUMBER:	
DATE MADE:	
EXP:	N/A

REAGENT	AMOUNT	LOT NUMBER	TICK WHEN ADDED
10XPBS	100mL		
DEIONIZED WATER	900mL		
Tween 20	0.5mL		

BLOCKING BUFFER/ASSAY DILUENT

1xPBS w/5% MILK

BATCH NUMBER:	
DATE MADE:	
EXP:	1 WEEK AFTER MADE:

REAGENT	AMOUNT	LOT NUMBER	TICK WHEN ADDED
10XPBS	100mL		
DEIONIZED WATER	900mL		
MILK POWDER	50g		

TMB SUBSTRATE SOLUTION

Prepare 6mL per plate working solution 15 minutes before it is needed. Mix equal volumes of Reagent A and B together and keep in the dark until needed.

REAGENT	AMOUNT	LOT NUMBER	TICK WHEN ADDED
REAGENT A	3mL per plate		
REAGENT B	3mL per plate		

STOP SOLUTION

2N H₂SO₄ or H₃PO₄

REAGENT	LOT NUMBER
H ₂ SO ₄	

COATING (PPD/85A):

Both needed at 1.5ug/mL. Coat with 50uL per well

<u>PPD FDA</u>	
LOT:	051815KA
STOCK CONCENTRATION:	0.5mg/mL
Coating Buffer per plate:	6mL
PPD per plate:	18uL

Aliquots made 7/7/15: 50uL per vial (enough for 4 plates)

<u>85A</u>	
LOT:	
STOCK CONCENTRATION:	0.82mg/mL
Coating Buffer per plate:	6mL
85A per plate:	10.87uL

Aliquots made 7/7/15: 80uL per vial (enough for 4 plates)

SECONDARY ANTIBODIES

Reconstitute antibodies with 1mL filtered, deionized water. Aeras recon 0.1mg of antibody in 1ml dioinised water giving conc of 0.1mg/mL. Need 6mL per plate. GOAT ANTI HUMAN SECONDARY – 1:500 (6ml per plate + 12uL of the Goat anti-human)

TITRATIONS

In separate non-treated 96-well V-bottom plate add 110uL of neat sample to wells (A-H row 1) according to plate map. Add 55ul sample diluent to dilution wells. Do a serial 1:2 dilution from row 1 to 12, taking 55uL of neat sample from row 1 into row 2, making sure to pipette up and down 4 times to thoroughly mix.

Transfer 50uL into ELISA plate when ready.

APPENDIX 4 - Total IgG calculations

TABLE 1 – 96-well plate layout with example duplicate data in rows A-G columns 1-12 (duplicate sample locations highlighted with boxes for columns 1-4 and row H). Total IgG standard curve dilutions and blanks are in row H, column 12 with concentrations detailed below row H. Data shown is OD reading at 450nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.328	1.745	1.233	1.384	1.464	1.248	1.484	1.265	1.350	1.470	0.998	1.100
B	1.300	1.667	1.217	1.384	1.494	1.194	1.466	1.272	1.407	1.346	0.954	0.950
C	1.242	0.770	1.089	1.197	1.197	1.638	1.246	1.321	1.210	1.318	0.734	1.080
D	1.261	0.748	1.011	1.183	1.220	1.591	1.120	1.207	1.143	1.318	0.707	1.130
E	1.193	1.276	1.223	1.506	1.530	1.288	1.120	1.100	0.931	1.418	1.379	1.290
F	1.129	1.317	1.159	1.460	1.413	1.186	1.062	1.025	0.916	1.461	1.358	1.350
G	1.453	1.381	1.504	1.469	1.029	1.011	1.445	1.393	1.492	1.565	1.130	1.110
H	1.326	1.278	0.916	0.920	0.638	0.567	0.319	0.384	0.249	0.231	0.086	0.070
	134.38ng/mL		67.19ng/mL		33.59ng/mL		16.8ng/mL		8.4ng/mL		0ng/mL	

TABLE 2 – Mean values of duplicate samples

	1	2	3	4	5	6	7	8	9	10	11	12
A	1.314	1.706	1.225	1.384	1.479	1.221	1.475	1.268	1.378	1.407	0.976	1.022
B												
C	1.251	0.759	1.049	1.190	1.208	1.614	1.181	1.263	1.176	1.318	0.720	1.105
D												
E	1.161	1.296	1.191	1.483	1.470	1.236	1.091	1.062	0.923	1.439	1.368	1.320
F												
G	1.417		1.486		1.020		1.419		1.528		1.120	
H												

TABLE 3 – Mean OD for standard curve concentrations

Standards concentration (ng/mL)	Mean Absorbance (OD)
134.38	1.302
67.19	0.918
33.59	0.603
16.80	0.352
8.40	0.240
0	0.078

Figure 1 – Standard curve fit

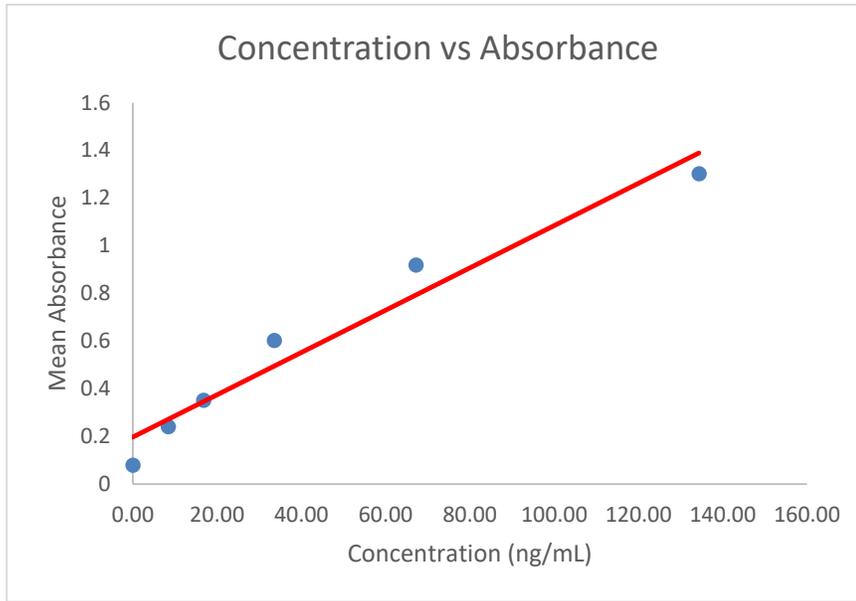


TABLE 4 – Total IgG ng/mL conversion using standard curve for duplicate measurements

	1	2	3	4	5	6	7	8	9	10	11	12
A	121.913	163.928	112.371	129.431	139.614	111.912	139.191	117.040	128.809	131.859	85.634	90.622
B												
C	115.212	62.372	93.522	108.616	110.597	154.141	107.687	116.419	107.119	122.350	58.237	99.469
D												
E	105.460	120.027	108.680	140.033	138.693	113.548	97.956	94.869	80.025	135.368	127.764	122.528
F												
G	132.922		140.416		90.377		133.160		144.886		101.104	

TABLE 5 – After linking sample locations to sample ID numbers, mean total IgG concentrations for test samples were multiplied by the dilution factor of 8×10^5 and converted into g/L by dividing by 1×10^6 .

Sample ID	Total IgG ng/mL	Dilution	CONCxDIL (ng/mL)	Total IgG (g/L)
843209	121.913	800000	97530674.432	97.5307
843227	163.928		131142015.250	131.142
843237	112.371		89896453.164	89.8965
843249	129.431		103544790.444	103.545
843254	139.614		111691589.431	111.692
843265	111.912		89529773.179	89.5298

APPENDIX 5 – Sodium thiocyanate antibody avidity calculations

TABLE 1 – 96-well plate layout of example data in rows A-H and columns 1-12 (duplicate samples are highlighted with boxes in columns 1-4). Column 1 and 2 contain the same sample diluted at 1:100. Row A contains 0M NaSCN, row B, 1M NaSCN and so on. Data shown is OD reading at 450nm.

NaSCN concentrations		1	2	3	4	5	6	7	8	9	10	11	12
0M	A	1.073	1.086	1.289	1.277	1.068	1.138	1.803	1.776	0.403	0.393	1.955	1.860
1M	B	0.270	0.282	1.063	1.077	0.415	0.433	1.626	1.599	0.387	0.381	1.270	1.230
2M	C	0.148	0.154	0.929	0.899	0.326	0.323	1.515	1.503	0.325	0.305	0.979	0.980
3M	D	0.108	0.115	0.588	0.615	0.212	0.228	1.265	1.279	0.263	0.265	0.653	0.650
4M	E	0.071	0.080	0.209	0.205	0.115	0.115	0.843	0.823	0.157	0.155	0.305	0.300
5M	F	0.054	0.051	0.091	0.088	0.068	0.071	0.239	0.259	0.043	0.038	0.105	0.110
6M	G	0.052	0.046	0.047	0.040	0.059	0.061	0.082	0.077	0.025	0.019	0.063	0.080
7M	H	0.052	0.049	0.036	0.035	0.049	0.046	0.059	0.062	0.020	0.017	0.073	0.070

TABLE 2 – Mean OD values for duplicate samples. Boxed cells A2, A4, A6, A8, A10 and A12 show the value which is half the maximum OD (eg A1/2).

NaSCN concentrations	1	2	3	4	5	6	7	8	9	10	11	12
0M	1.080	0.53975	1.283	0.6415	1.103	0.5515	1.790	0.89475	0.398	0.199	1.908	0.95375
1M	0.276		1.070		0.424		1.613		0.384		1.250	
2M	0.151		0.914		0.325		1.509		0.315		0.980	
3M	0.112		0.602		0.220		1.272		0.264		0.652	
4M	0.076		0.207		0.115		0.833		0.156		0.303	
5M	0.053		0.090		0.070		0.249		0.041		0.108	
6M	0.049		0.044		0.060		0.080		0.022		0.072	
7M	0.051		0.036		0.048		0.061		0.019		0.072	

TABLE 3 – After linking sample locations to sample ID numbers, avidity Index (AI) was calculated as the molar concentration of NaSCN required to reduce the measured OD to half of the maximum.

Sample ID	Avidity Index (AI)
901362	1.950
900378	2.981
906591	2.027
904643	3.603
902932	3.511
906595	2.509

RESEARCH ARTICLE

Human cytomegalovirus epidemiology and relationship to tuberculosis and cardiovascular disease risk factors in a rural Ugandan cohort

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Abstract

Human cytomegalovirus (HCMV) infection has been associated with increased mortality, specifically cardiovascular disease (CVD), in high-income countries (HICs). There is a paucity of data in low- and middle-income countries (LMICs) where HCMV seropositivity is higher. Serum samples from 2,174 Ugandan individuals were investigated for HCMV antibodies and data linked to demographic information, co-infections and a variety of CVD measurements. HCMV seropositivity was 83% by one year of age, increasing to 95% by five years. Female sex, HIV positivity and active pulmonary tuberculosis (TB) were associated with an increase in HCMV IgG levels in adjusted analyses. There was no evidence of any associations with risk factors for CVD after adjusting for age and sex. HCMV infection is ubiquitous in this rural Ugandan cohort from a young age. The association between TB disease and high HCMV IgG levels merits further research. Known CVD risk factors do not appear to be associated with higher HCMV antibody levels in this Ugandan cohort.

Introduction

Human Cytomegalovirus (HCMV), also known as human herpesvirus-5 (HHV-5), is a member of the β -herpes virus family which is widely distributed in human populations. HCMV transmission occurs through person-to-person contact. It can be transmitted transplacentally to neonates or through breast milk of an infected and shedding mother, by intimate contact and by transplantation from (or sharing syringes with) an infected individual [1]. It has been shown that young children shed HCMV virus in saliva and urine at high levels which may add to transmission between infants and adult caregivers [2].

Congenital HCMV infection is the leading cause of permanent hearing and neurological impairment as well as vision loss in infants worldwide [3]. Maternal primary infection or

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reactivation, especially during the first trimester, is particularly associated with adverse neonatal outcomes [4]. The incidence of congenital HCMV infection is estimated at between 0.7 and 5% of all births in low- and middle-income countries (LMICs) [5].

In immunocompetent adults, HCMV infection rarely causes disease; however, once infected, the virus remains latent in a wide range of cell types, including lymphocytes and myeloid lineage cells, as well as smooth muscle cells and endothelial cells which line blood vessels [6]. HCMV/HIV co-infection is common and is an important cause of HCMV retinitis and severe non-AIDS events, including death, in HIV-infected individuals [7,8]. HCMV infection is associated with chronic immune activation [9] and recent evidence implicates immune activation with increased risk of tuberculosis (TB) disease [10].

Epidemiological studies in high-income countries (HICs) have found associations between HCMV infection and increased risk of mortality in older people [11,12]. Further studies have implicated chronic HCMV infection as a risk factor for cardiovascular disease (CVD); a recent meta-analysis of studies conducted in HICs, estimated a 22% increased relative risk of CVD with exposure to HCMV [13]. In a UK setting, HCMV infection was associated with the development of arteriosclerosis [14] and a 3mmHg increase in systolic blood pressure among older individuals [15]. As Africa undergoes what has been described as an 'epidemiological shift' from infectious to non-communicable disease [16], an estimated 1.2 million deaths in Africa were attributed to CVD in 2004 [17].

Associations of HCMV and CVD have not been fully investigated in LMICs where HCMV infection rates are much higher than in HICs [18–20]. Studies from other herpes viruses associated with non-communicable disease (NCD) in areas where infection is ubiquitous (such as EBV in relation to African Burkitt lymphoma and KSHV in relation to African Kaposi Sarcoma), show that risk of NCD increases with increasing viral antibody titre [21–24].

In this study, we investigate HCMV seroprevalence in a large cross-sectional rural Ugandan cohort (n = 2,174) and investigate associations with co-infections, clinical measurements and demographic information. As a secondary analysis, we link cardiovascular risk factors to HCMV antibody levels.

Material and methods

Study area and design

The General Population Cohort (GPC) is a population-based open cohort study, set up in 1990 by the Medical Research Council (MRC) UK in collaboration with the Uganda Virus Research Institute (UVRI). Initially to examine trends in HIV prevalence and incidence, the GPC is located in Kyamulibwa sub-county of Kalungu district, rural south-western Uganda [25]. The cohort now comprises a cluster of 25 neighboring villages with approximately 20,000 residents (52% aged ≥ 13 years) from three ethnic groups, the majority (75%) being from the Baganda tribe, the main tribal group in the region.

Blood samples are transported to MRC/UVRI laboratories in Entebbe where a portion of the venous blood sample is analysed according to protocol guidelines. Remaining samples are stored at -80°C in a biobank in Entebbe [25].

The majority of samples tested for HCMV in this study were taken from 2011. Samples from TB cases were from a range of GPC rounds sampled between 1999 and 2014. Active pulmonary TB was diagnosed through positive sputum smear microscopy after passive case detection.

Sampling

Individuals were selected at random, having been stratified by age and sex. Infants under 5 years of age were oversampled in anticipation of high age-dependent HCMV seropositivity

based on evidence from other sub-Saharan countries. A target sample size of approximately 100 individuals per year of age from under 1 year to 5 year were sampled and 200 individuals in 5 to 10 year age groups thereafter. The sex ratio was approximately equal within each age group (Table 1).

The age structure of sampling for this study was determined based on Ugandan demographics; 49% of Ugandans are under 14 years of age, 21% are between 15–24 years of age, 28% between 25 and 64 years of age and only 2% over 65. The sampling here was similar to the Ugandan demographic with 45% under 14 years, 18% 15–24, 32% 25–64 and 5% over 65 years of age.

Being a cross-sectional study, each individual was only sampled once. Siblings and parent-child pairs were not excluded.

Ethics

Written consent for the use of clinical records and biological samples for research purposes was obtained from all GPC participants following Uganda National Council of Science and Technology guidelines.

Ethical approval for the use of samples for this study was obtained from The UVRI Research and Ethics Committee and from the Uganda Council for Science and Technology, in addition to the London School of Hygiene & Tropical Medicine, London, UK.

HCMV serology

Serum samples were tested for antibodies against HCMV using standard, validated and commercially available enzyme-linked immunosorbent assay (ELISA) against HCMV IgG (IBL

Table 1. General population cohort characteristics of individuals included in this study.

Age, mean (range)	22.7 (0.08–100.75)
Sex, percentage female (number/total)	50.2% (1,091/2,174)
HIV prevalence, percentage (number/total)	4.6% (100/2,134)
Body mass index*, percentage (number/total)	
- Underweight	21% (237/1,150)
- Normal	69% (797/1,150)
- Overweight	8% (97/1,150)
- Obese	2% (19/1,150)
Hypertension**, percentage (number/total)	
- Normal	46% (552/1,187)
- Pre-hypertension	39% (460/1,187)
- Hypertension	15% (175/1,187)
Tribe, percentage (number/total)	
- Baganda	75% (1,524/2,035)
- Rwandese Ugandan	16% (319/2,035)
- Other §	9% (192/2,035)

*body mass index 18.4 and below is underweight, 18.5–24.9 is normal, 25.0–29.9 is overweight, and 30 and above is obese.

** Pre-hypertension is systolic blood pressure (SBP) between 120mmHg and 140 mmHg, and a diastolic blood pressure (DBP) between 80 mmHg and 90 mmHg. Hypertension is defined as SBP \geq 140mmHg and DBP \geq 90mmHg.

§ Other tribes include Bakiga, Batooro, Banyankole, Basoga, Bafumbira, Tanzanian, Barundi

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International GmbH). Testing was conducted at UVRI in Entebbe. Briefly, serum samples were thawed and diluted as per kit instructions to 1:100 in sample diluent. One hundred microliters of diluted serum and controls in duplicate were dispensed into a 96-well plate coated with HCMV antigen. Plates were covered and incubated for 1hr at 37°C and then washed before 100µL of anti-IgG conjugate was added and the plate covered and incubated again for 30 minutes at room temperature. After another washing step, 100µL of tetra-methylbenzidine (TMB) substrate solution was added and the plate incubated for 15 minutes in the dark at room temperature before 100µL of 0.2M sulphuric acid solution was added to stop the reaction. Plates were read at 450/620nm within 30 minutes to obtain optical density (OD), a surrogate marker of antibody titer.

Individuals were considered to be seropositive if the IgG OD measurement of plate controls fell within the kit specifications and the mean of duplicate measurements was above the calculated cut off.

Linking to GPC data

Testing for HIV was carried out immediately after blood collection in Uganda as previously described by Asiki *et al* [25]. Data were linked to the samples retrieved via each participant's unique GPC identifier.

Anthropometric measurements and blood sampling were performed by trained interviewers/nurses using calibrated instruments following standard protocol guidelines. Body mass index (BMI) was calculated as weight (kg) divided by height (m)². Pre-hypertension was defined as having a systolic blood pressure (SBP) between 120mmHg and 140 mmHg, and a diastolic blood pressure (DBP) between 80 mmHg and 90 mmHg. Hypertension was defined as having a SBP \geq 140mmHg and DBP \geq 90mmHg. Proportion hypertensive was calculated by dividing the number of hypertensive individuals by the combined number of normal and pre-hypertensive individuals. Classification of BMI into four categories and definition of hypertension were derived from current World Health Organization (WHO) and National Institute of Health guidelines, respectively [26,27]. Biochemical analyses were performed using the Cobas Integra 400 plus analyser to determine lipid profiles for total cholesterol, high and low density lipoproteins (HDL and LDL respectively) from serum samples, and HbA1c from whole blood samples. Abnormal lipids were defined as raised total cholesterol being $>$ 5.2mmol/L, low HDL as $<$ 1.0mmol/L for men, and $<$ 1.3mmol/L for women [28].

Total IgG was measured by ELISA utilizing a commercial standard curve. Degraded samples with negative total IgG were excluded from the study.

Samples used in this study were linked to demographic and other clinical data corresponding to the same time period at which the sample was taken.

Statistical analysis

Participants were categorized as either HCMV seronegative or seropositive based upon OD results; seropositive samples were further categorized into tertiles of OD, to differentiate low, medium and high responders. The Student's t test was used to compare continuous variables (such as HCMV IgG antibody OD measurement) between two independent groups (sex, tribe (Baganda or other), HIV and TB status). Associations of HCMV IgG OD with age, tribe, sex, HIV and TB were investigated. An analysis of variance (ANOVA) was used to compare continuous variable outcome between HCMV tertiles.

Linear regression was used to determine the relationship between HCMV IgG OD (as the dependent variable) and sex, HIV and TB status (as the independent variables—tribe was excluded in HCMV IgG OD regression analysis due to missing data— 10/27 active TB cases did

not have information on tribe), and also between CVD risk factors with a continuous measurement (dependent variables: HbA1c, cholesterol, HDL, LDL and BMI) and the independent variables HCMV tertile, age and sex. Logistic regression was used for the binary outcome variables HCMV seropositive/seronegative and proportion hypertensive. Due to the approximate two-fold increase in relative risk of coronary heart disease or acute myocardial infarction in HIV-infected patients [29–31], associations between HCMV and CVD variables are limited to HIV negative individuals in this analysis.

HCMV OD data were slightly positively skewed, possibly resulting in artificially narrower confidence intervals (CI) around estimates. Cardiovascular disease (CVD) risk measurements were available for individuals over the age of 12 years.

To account for evidence of non-linearity of HCMV OD with age (likelihood ratio test $p = 0.0025$), a quadratic term for age was included in the regression analyses.

To account for multiple comparisons, 99% CI are reported and a p value of 0.01 is considered to represent strong evidence to reject the null hypothesis.

Data were entered using Microsoft Excel and analysed using STATA version 14 (Stata Corporation, College Station, TX, USA).

Results

Of the 2,189 samples tested, 15 had negative total IgG levels and so were excluded due to probable degradation of sample, resulting in 2,174 samples being included in initial analyses. Following these exclusions, 40 individuals did not have a valid HIV result and so were excluded from regression analysis where HIV was included as a regression term.

Overall 91% (1,988/2,174) of the population tested positive for HCMV antibodies. Among children less than 15 years of age, 1% (10/991) were HIV positive; among individuals over 15 years of age, 8% (90/1,143) were HIV positive. The highest proportion infected with HIV (15%) was within the 41–50 year age group.

Factors affecting HCMV IgG antibody levels

The percentage of HCMV seropositive individuals in this population increases for both males and females equally from birth until the 6–10 year age group, after which seropositivity levels plateau at around 95% (Fig 1): 83% HCMV seropositivity was seen by age one year and this increased to 95% by age five.

Because of the ubiquity of HCMV infection within this population it was decided to conduct further analyses on the HCMV seropositive population only. An analysis of the HCMV negative population ($n = 186$, none of whom were TB cases and only 4 HIV positive) showed no significant associations for sex or HIV in regression analysis. The only significant association was with age in years ($p < 0.001$).

Of the HCMV seropositive individuals, IgG levels initially decrease from a peak in those < 1 year until the 16–20 year age group, after which HCMV IgG levels increase for both males and females (Fig 2A). When HIV negative individuals are plotted separately, the increased HCMV IgG levels in the 31–50 year old groups coincide with the highest HIV positivity (Fig 2B).

To investigate impact of age on HCMV IgG levels, ages were further split into < 9 months, 9 months to 12 years, 13–20 years, 20–60 and 60 years and above. Age groups selected are based on presence of maternal antibodies (conservative estimate of 9 months [32]), prior to sexual debut (9 months—12 years [33]), post sexual debut (13–20 years [33]), 21–59 years and post 60 years. It was found that infants under the age of 9 months have higher mean HCMV IgG (1.25 OD) than any other age group apart from the over 60 years olds (1.34 OD) in unadjusted analyses.

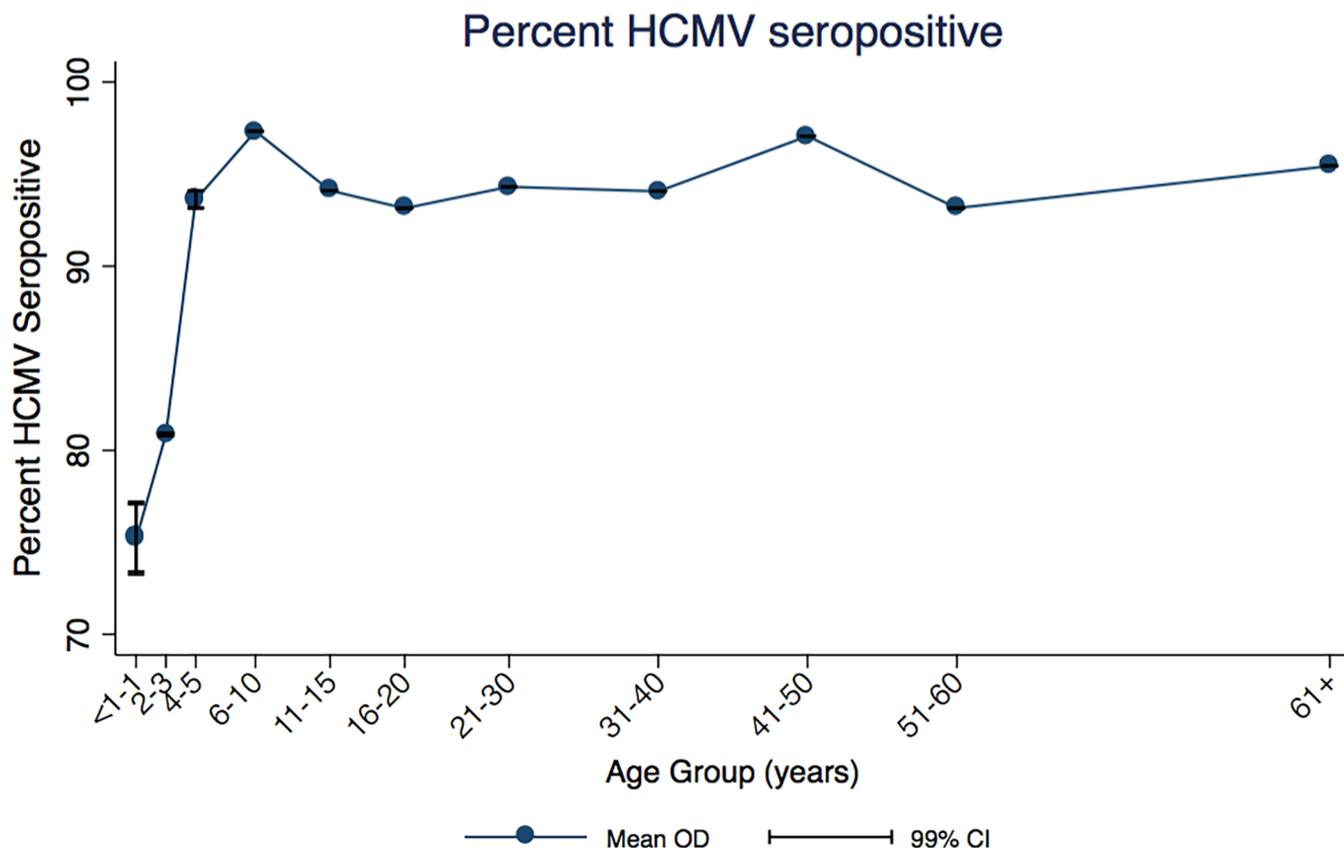


Fig 1. Percent HCMV seropositive by age group (years). Vertical lines show 99% CI for each mean data point. CI—confidence interval, HCMV—human cytomegalovirus.

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A 0.07 OD increase in HCMV IgG (99% CI 0.02,0.12, $p < 0.001$) is associated with being female in unadjusted analyses. This increase remained after adjusting for age, HIV and TB infection status (mean difference 0.07 OD 99% CI 0.03,0.11, $p < 0.001$) (Table 2). The 0.49 OD increase in HCMV IgG associated with HIV infection also remained after adjustment for age, sex and TB infection status (mean difference 0.47 OD 99% CI 0.37,0.57, $p < 0.001$) (Table 2).

Mean HCMV IgG OD levels among the 27 people with sputum-confirmed pulmonary TB was 0.34 OD higher than individuals without active TB. After adjusting for age, sex and HIV infection status this difference is partly explained by those variables but there remains good evidence of that active TB disease is associated with an increased HCMV level of 0.19 OD (99% CI 0.01,0.37, $p = 0.006$) (Table 2) which equates to a 7% increase based the range of HCMV OD values (min 0.31, max 2.84 OD). When HCMV seropositivity was divided into low, medium and high responders, the majority of active TB cases had high HCMV IgG levels (16/27, 59%). Thirty three percent were of medium HCMV IgG (9/27), 7% (2/27) had low HCMV, and none were HCMV seronegative (Fig 3).

When HIV positive and negative individuals are analysed separately, the effect of active TB disease is shown to be greater in the HIV positive group, but the direction of effect is the same. Among HIV negative people (S1 Table), having active TB disease is associated with an adjusted increase of 0.11 OD of HCMV IgG (99% CI -0.10–0.33, $p = 0.170$) whereas among the HIV positive individuals (S2 Table), having active TB disease is associated with an increased HCMV IgG OD of 0.32 OD (99% CI -0.12,0.77, $p = 0.063$).

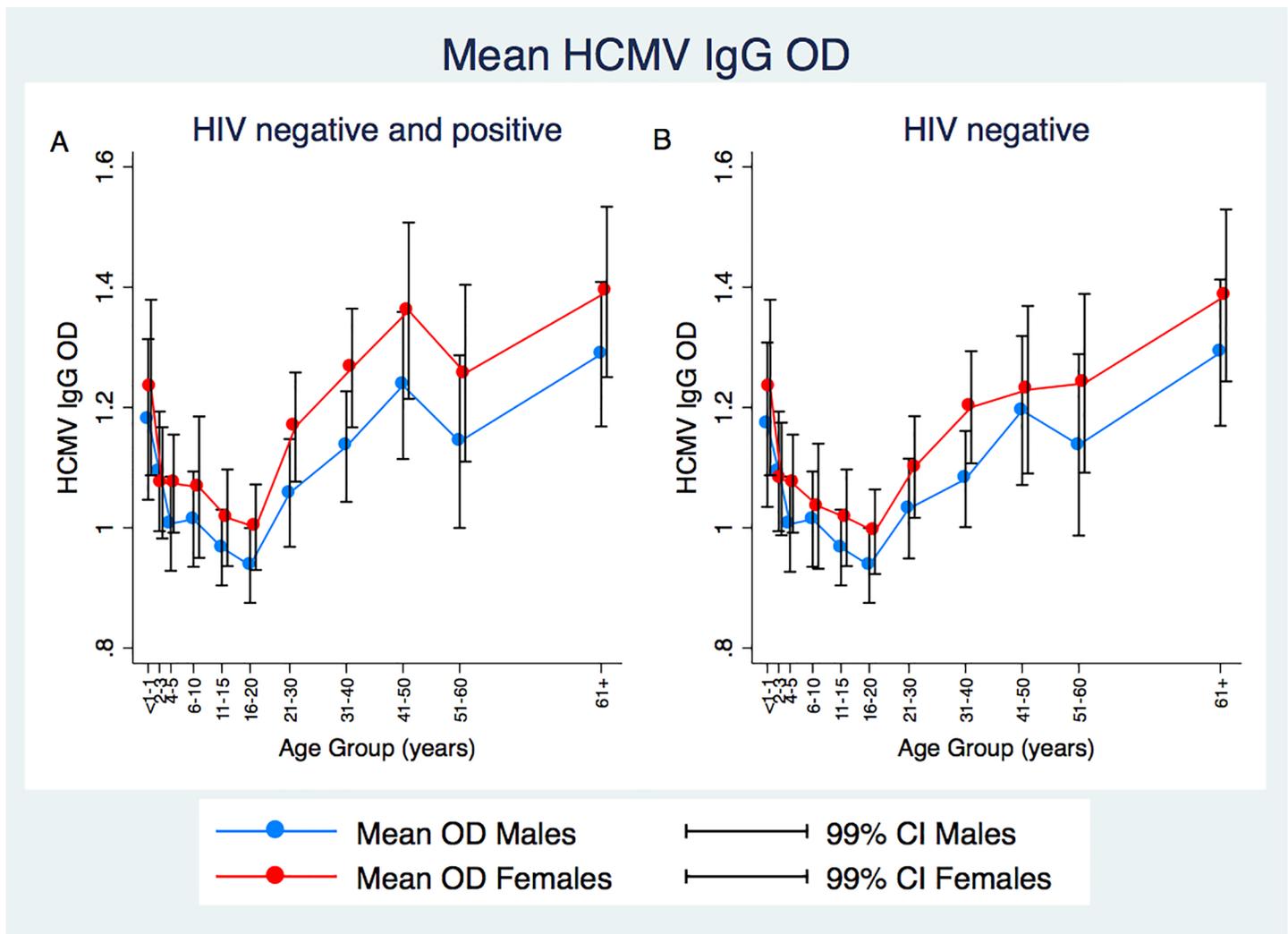


Fig 2. Fig 2A. Mean HCMV IgG OD levels in HIV negative, HCMV seropositive individuals by age group (years) and sex. Fig 2B. Mean HCMV IgG OD levels in HIV negative and HIV positive individuals combined by age group (years) and sex. Red lines show female mean values, blue shows male mean values. Vertical lines show 99% CI for each mean data point. CI—confidence interval, HCMV—human cytomegalovirus, HIV—human immunodeficiency virus.

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Being from a tribe other than the majority Baganda tribe was associated with a 0.14 OD increase in HCMV IgG (99% CI 0.09,0.19, $p < 0.001$). Due to missing data (10/27 active TB cases did not have information on tribe), and the fact that inclusion of tribe into regression analyses did not alter mean difference coefficients, this variable was not included in regression analyses. Although not included, a sensitivity analysis resulted in a change in coefficient for sex from 0.068 to 0.070 OD, a change in coefficient for hiv from 0.466 to 0.454 and a change in coefficient for TB from 0.193 to 0.184. The coefficient for tribe was 0.11 OD 99% CI 0.06–0.16.

Association of HCMV IgG levels with cardiovascular disease risk factors

Cardiovascular risk factors were assessed in individuals over 12 years of age (mean age of individuals included in CVD risk factors analysis: 36.5 years, range 12–100 years) due to availability of data.

Table 2. Unadjusted and fully adjusted mean differences (values obtained using a multivariable model including age, quadratic age, sex, HIV and TB status) in HCMV IgG OD with p value (t test for unadjusted values, regression for adjusted values) and 99% confidence intervals.

Factor (n)	HCMV IgG					
	Unadjusted mean difference	P value	99% CI	Adjusted mean difference	P value	99% CI
Sex						
Male (978)	baseline			baseline		
Female (978)	0.07	<0.001	0.02, 0.12	0.07 §	<0.001	0.03, 0.11
HIV †						
Negative (1,860)	baseline			baseline		
Positive (96)	0.49	<0.001	0.40, 0.60	0.47	<0.001	0.37, 0.57
TB ‡						
Negative (1,929)	baseline			baseline		
Positive (27)	0.34	<0.001	0.15, 0.53	0.19	0.006	0.01, 0.37

CI—confidence interval, HCMV—human cytomegalovirus

†HIV—human immunodeficiency virus

‡TB—Active pulmonary Tuberculosis.

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Because of the known increased risk of CVD in HIV-infected individuals, associations between HCMV and CVD predictors is limited to HIV negative individuals in this analysis, who represented 94% (2,034/2,174) of the total sample.

Of the 2,034 HIV negative individuals included in this analysis, data for cholesterol, HDL, LDL, and mean SBP and DBP were available for 58% (1,186) of individuals, HbA1c levels for 58% (1,176) and BMI measurements were available for 57% (1,150) of individuals, all of whom were over the age of 12 years.

Among the individuals for whom BMI data was available in this cohort, 21% (237/1,150) were underweight, 69% (797) normal weight, 8% (97) overweight and 2% (19) obese. Of the 1,0186 individuals with measured blood pressure, 15% (175) were hypertensive, 39% (460) were pre-hypertensive, and 46% (552) were not hypertensive (Table 1).

Initial regression analyses showed no evidence of a difference between CVD risk factors between HCMV seropositive and HCMV seronegative individuals in a model adjusting for age and sex. When HCMV seropositive individuals were further divided into low, medium and high responders, the only CVD risk factor that showed an association with HCMV IgG levels in univariate analysis was hypertension, which is more prevalent in people with medium or high HCMV IgG levels (Fig 4). After adjusting for age and sex however, this association is explained by these variables (Table 3). Addition of tribe as an explanatory variable in regression analyses did not alter coefficients.

Discussion

HCMV is an important pathogen in congenital neurological conditions and in the context of immune suppression. Recent studies have suggested a link between HCMV seropositivity and excess overall mortality, specifically from CVD, within HIC settings [12–15,34]. Relatively little attention has been paid to determining HCMV prevalence and associations with non-communicable disease in LMICs.

Here, we find that 95% of this rural Ugandan population are seropositive for HCMV by age five years (Fig 1). This finding corroborates other findings in sub-Saharan African settings where high levels of HCMV seropositivity have been found; 96% in Egypt [35], 97% in Benin [36], 99% in the Gambia [37] and 86% in South Africa [38].

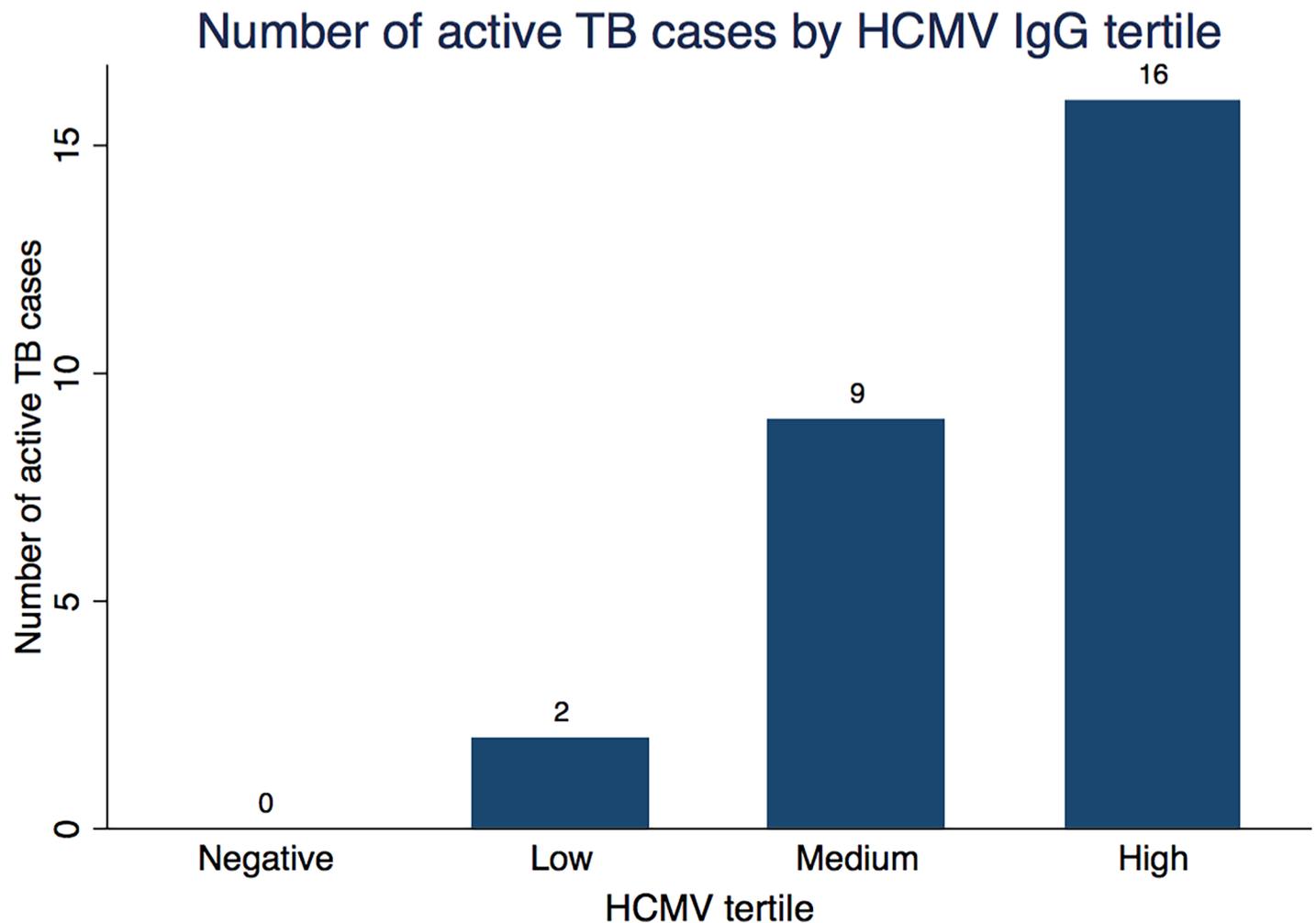


Fig 3. Number of active TB cases by HCMV IgG tertile. Numbers above bars show number of active TB cases (n = 27 total active TB cases).

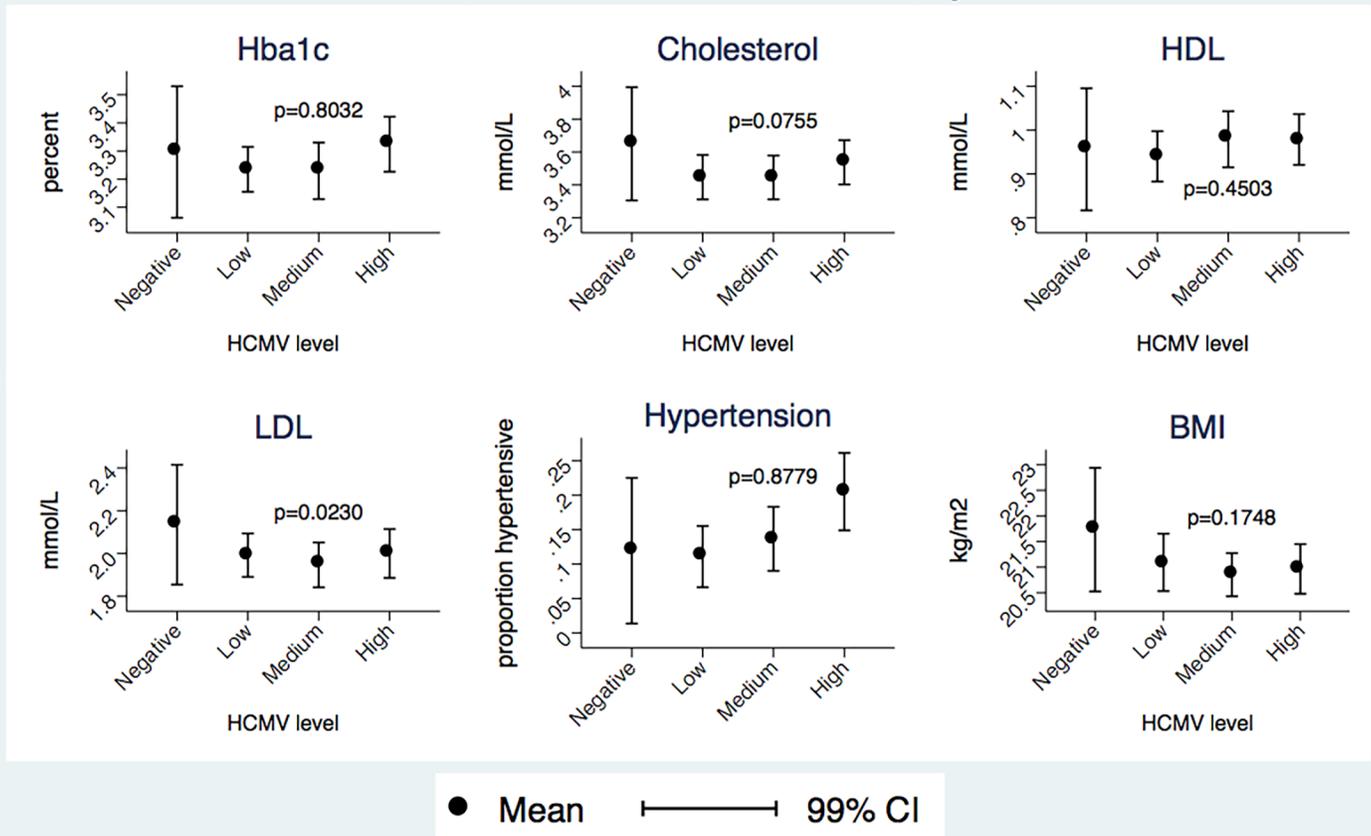
<https://doi.org/10.1371/journal.pone.0192086.g003>

Among the HCMV seropositive population in this study we see high levels of HCMV IgG antibody in young infants under the age of nine months (Fig 2). While this may be a true reflection of congenital or primary infection of infants, IgG is the only antibody isotype that can be transferred through the placenta [39], and so these high levels may be driven by the inability of the assay to distinguish between maternal and 'self' IgG. Detection of virus as opposed to antibodies would allow elucidation during the first nine months of life while maternal antibodies persist.

After initial high levels of HCMV IgG in infants under the age of one year, we see a rapid decline whereby maternal antibodies are lost, or primary infection is controlled (but individuals remain seropositive), followed by a gradual increase from the age of 16 years of age up to the highest OD levels seen after 40 years of age.

The magnitude of antibody response to virus after maternal antibodies have waned is indicative of intensity of exposure. The finding that females have higher HCMV antibody levels compared to males after adjusting for age, HIV and TB (Table 2) was surprising. There is evidence that risk factors throughout life result in a higher risk of HCMV transmission in women compared to men; a study conducted in Uganda using data on breast feeding trends, showed

Cardiovascular Disease Risk Factors by HCMV tertile



Unadjusted mean values, percentage or proportions plotted with age and sex adjusted p values from a likelihood ratio test

Fig 4. Bar graphs showing unadjusted mean CVD risk factors by HCMV IgG OD level. HIV positive individuals were excluded from this analysis. Mean and 99% CI with p value from linear regression or logistic regression (for hypertension). p values shown are from age- and sex-adjusted likelihood ratio tests. CI—confidence interval, CVD—cardiovascular disease, HCMV—human cytomegalovirus, HIV—human immunodeficiency virus.

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Table 3. Age and sex-adjusted regression for CVD risk factors cholesterol, HbA1c, HDL, LDL and BMI and hypertension showing mean difference (or Odds Ratio), p value from likelihood ratio test and 99% CI.

HCMV level	HbA1c (%)			Cholesterol (mmol/L)			HDL (mmol/L)			LDL (mmol/L)			Hypertension (proportion)			BMI (mg/kg2)		
	Mean difference	p value	99% CI	Mean difference	p value	99% CI	Mean difference	p value	99% CI	Mean difference	p value	99% CI	Odds ratio	p value	99% CI	Mean difference	p value	99% CI
Negative	baseline			baseline			baseline			baseline			baseline			baseline		
Low	-0.032	0.803	-0.26, 0.20	-0.113	0.076	-0.43, 0.20	0.007	0.450	-0.14, 0.15	-0.080	0.023	-0.34, 0.18	1.073	0.878	0.34, 3.35	-0.485	0.175	-1.69, 0.72
Medium	-0.072		-0.30, 0.16	-0.172		-0.49, 0.14	0.025		-0.12, 0.17	-0.159		-0.41, 0.10	1.095		0.36, 3.34	-0.736		-1.93, 0.46
High	-0.046		-0.28, 0.19	-0.266		-0.58, 0.05	-0.028		-0.17, 0.12	-0.234		-0.49, 0.02	1.256		0.42, 3.78	-0.910		-2.12, 0.30

CI—confidence interval, CVD—cardiovascular disease, HDL—high density lipoproteins, LDL—low density lipoproteins, BMI—body mass index

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that being a male baby increased the risk of early termination of breastfeeding compared to females [40]. Findings from another Ugandan longitudinal cohort found that age at sexual debut has been consistently lower for women than for men from the 1950's to 1990's [33]. In addition, the infant caregiving role of females results in disproportionate female interaction with infants during the peak shedding at 1–2 years of age [41].

Our results suggest that HIV infection and active TB disease are independently associated with increased levels of HCMV IgG antibodies (Table 2). HIV infection is correlated with hypergammaglobulinaemia by way of defective humoral immunity resulting in hyperactivated naive B cells producing large quantities of IgG [42]. Here we see an association between HIV infection and elevated IgG specific to HCMV, however in the same cohort, we see increased total IgG as well as tetanus toxoid-specific IgG in HIV-infected individuals, an association which remained after adjusting for age and sex (Stockdale *et al.*, manuscript in preparation). Despite this potential confounding of HIV infection upon IgG levels, the increased levels of HCMV IgG in HIV infected individuals is corroborated by epidemiological evidence pointing towards high levels of HIV and HCMV co-infection due to similar routes of transmission [7].

Both TB and HIV are known to increase general inflammation [43,44], an immune environment which is associated with the increased likelihood of HCMV reactivation [45]. Both reactivation and reinfection expose the immune system to HCMV antigens, thereby resulting in elevated levels of HCMV-specific IgG antibodies.

The association of active pulmonary TB with raised HCMV IgG levels was in contrast to findings of a hospital-based study where HCMV-specific T-cell responses in a whole blood assay were found to be lower in TB patients who died when compared to TB patients who survived [46]. In this hospital-based study, HCMV responses were likely a general indicator of 'immune fitness' and therefore predictive of patient survival. By contrast, our study was community based and the severity of TB disease at the time of blood draw would have been low when compared to a hospital-based study.

The association of active pulmonary TB with raised HCMV IgG in our study was consistent with the findings of a correlates of TB risk study in South African infants where risk of active TB disease was increased with an activated T-cell phenotype which was itself correlated with T-cell interferon gamma production upon stimulation with HCMV antigens [10]. Neither active case finding, nor routine latent TB infection screening is conducted in the Ugandan GPC and therefore we cannot be certain that 'non-TB' individuals here have not been exposed to TB or indeed that they are not latently infected with *Mycobacterium tuberculosis*. Despite there only being 27 active TB cases within this cohort, there remains strong evidence of an association between TB and elevated HCMV IgG after adjusting for age and sex; an association which appears to be intensified by co-infection with HIV (S1 and S2 Tables).

In view of the 1.5 million deaths per year associated with TB [47], the association of increased HCMV IgG with active TB disease may be an important future research area, especially in areas with large numbers of people living with HIV.

It is important to note that the data from this study gives us no information on causality. Longitudinal studies measuring systemic viral loads along with antibody levels to HCMV (and other herpes viruses) in stored sera would be useful to understand progression of both herpes virus and TB disease in this cohort.

Association of HCMV with CVD risk factors

Rates of CVD are increasing in sub-Saharan Africa [48]. In 2014, the WHO estimated that 27% of all deaths in Uganda were attributable to non-communicable diseases; specifically 9% of all deaths to CVD [49]. In HICs it has been postulated that the increased overall mortality

associated with HCMV infections is associated with vascular morbidity [34]. HCMV infection has been implicated in increased arterial stiffness in chronic kidney patients [14] and in older individuals in the UK [15], and there is growing evidence of an important role for HCMV-induced inflammation in vessel walls [50] through persistent replication of HCMV in endothelial cells [6].

The current literature reports conflicting evidence for an association of HCMV infection with increased risk for CVD mortality. Of the ten studies included in a recent meta-analysis which found an excess risk of ischemic heart disease, stroke, and cardiovascular death with HCMV exposure [34], none were carried out in sub-Saharan Africa or other LMICs (studies were from United States (4 studies), United Kingdom (3 studies), Canada (1 study), Sweden (1 study), and Italy (1 study)). Another large meta-analysis comprising three studies and 9,657 patients found an overall significant association between HCMV positivity and hypertension (79% of the hypertension patients were HCMV positive compared with 64% of the controls—(Odds Ratio (OR) 1.39, 95% CI 0.95,2.05, $p = 0.017$) [13]. Of the three studies included, one was carried out in the USA, one in Iran and one in China. The overall OR masks large heterogeneity in the data, with the Iranian study showing high levels of HCMV seropositivity (93% overall seropositivity) and no association, while the US study reported 55% HCMV seropositivity and association between HCMV and hypertension among males only [13]. In one US population, association of HCMV seropositivity with all-cause mortality remained after adjusted analysis, however specific association with CVD mortality disappeared after adjusting for confounders [51] and in another, an association with hypertension was largely explained away by age [52].

The aforementioned studies investigated HCMV seropositivity in relation to CVD risk factors. In our study we investigate magnitude of antibody response to HCMV in a near-ubiquitously infected population. We do not see an association between the burden of HCMV infection, as measured by IgG, and CVD risk factors. It may be that the association between HCMV and CVD is masked due to burden of other chronic and acute infections in these communities. However more studies are needed to determine any association with excess mortality in populations with ubiquitous HCMV infection, and to assess if risk factors found to be important in one population are useful indicators of risk in other populations. Indeed, many studies have found reference standards developed in Europe and North America unhelpful when studying other populations [53,54]. It is essential that measurements are clinically relevant to the population in question, and that clinical research institutions ensure that standardized methods are used if data are to be comparable across countries and continents.

Despite the young age of individuals in this cohort (mean age 36.5 years, range 12–100 years), we do know that CVD outcomes (such as stroke) are not uncommon [55] and so these analyses could be expected to show association with HCMV antibodies if any exist. Evidence from a large cohort study in the US found that CVD risk factors linked to myocardial infarction and coronary heart disease death in later life are measurable in individuals as young as 20 years of age [56].

Despite an established link between HCMV serum antibody levels and blood viremia (and viral shedding in urine and saliva) among children [57], and the link between high systemic viral copy number and increased risk of mortality in HIV positive adults [58], it may be of interest in future studies to link viral load and risk factors to active HCMV disease in this population. Due to limited sample volume here, we were unable to measure viral DNA copy number in this study.

Considering the paucity of studies linking HCMV and all-cause mortality in LMIC settings and the association seen here between HCMV and TB, we believe it will be important to

conduct longitudinal studies to elucidate these associations further in areas with almost universal prevalence of HCMV infection, specifically in sub-Saharan Africa.

Limitations

The GPC has no official vital registration system and so we do not have CVD outcomes to link to CVD risk factors. Future studies which specifically investigate CVD related deaths within the GPC would be helpful to link HCMV antibody levels in longitudinal retrospective analyses using stored sera. The GPC is perfectly placed to collect CVD outcome data in future rounds of data collection and so these outcome measures can be linked back to clinical measurements and stored biological samples.

Supporting information

S1 Table. Unadjusted and fully adjusted mean differences (values obtained using a multi-variable model including age, quadratic age, sex and TB status) in HCMV IgG OD with p value (t test for unadjusted values, regression for adjusted values) and 99% confidence intervals for HIV negative individuals only (n = 1,860).

(DOCX)

S2 Table. Unadjusted and fully adjusted mean differences (values obtained using a multi-variable model including age, quadratic age, sex and TB status) in HCMV IgG OD with p value (t test for unadjusted values, regression for adjusted values) and 99% confidence intervals for HIV positive individuals only (n = 96).

(DOCX)

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HIV, HCMV and mycobacterial antibody levels: a cross-sectional study in a rural Ugandan cohort

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Abstract

OBJECTIVES A growing evidence base implicates human cytomegalovirus (HCMV) as a risk factor for TB disease. We investigated total IgG and mycobacteria-specific antibodies in a cross-sectional study nested within a rural Ugandan General Population Cohort (GPC), in relation to HIV infection and the magnitude of HCMV IgG response.

METHODS Sera from 2189 individuals (including 27 sputum-positive TB cases) were analysed for antibodies against mycobacteria (Ag85A, PPD, LAM, ESAT6/CFP10) and HCMV, tetanus toxoid (TT) and total IgG.

RESULTS Anti-mycobacterial antibodies increased with age until approximately 20 years, when they plateaued. Higher HCMV exposure (measured by IgG) was associated with lower levels of some anti-mycobacterial antibodies, but no increase in total IgG. HIV infection was associated with a decrease in all anti-mycobacterial antibodies measured and with an increase in total IgG.

CONCLUSIONS The increase in anti-mycobacterial antibodies with age suggests increasing exposure to non-tuberculous mycobacteria (NTM), and to *M.tb* itself. HIV infection is associated with decreased levels of all mycobacterial antibodies studied here, and high levels of HCMV IgG are associated with decreased levels of some mycobacterial antibodies. These findings point towards the importance of humoral immune responses in HIV/TB co-infection and highlight a possible role of HCMV as a risk factor for TB disease.

keywords TB, HIV, HCMV, *Mycobacterium*

Introduction

The role of antibody-mediated immunity in TB has not been fully elucidated (reviewed in [1]). Despite the importance of cell-mediated Th1 responses in TB disease [2, 3], attempts to stimulate this arm of the human immune response by vaccine developers have not translated into protection from disease in human efficacy trials [4, 5], and there may be useful humoral correlates of disease or protection yet to be identified.

A growing evidence base for the importance of antibodies in protection against other intracellular pathogens such as *Listeria monocytogenes* [6] and *Salmonella* spp. [7] has led to renewed interest in humoral responses to

TB and the factors which may affect them. Recent work has suggested a potential protective role of antigen 85A (Ag85A)-specific antibodies in reduced TB disease risk in BCG-vaccinated South African infants [8, 9].

It is known that TB and HIV work synergistically to exacerbate morbidity and mortality in co-infected individuals with respect to both diseases [10]. Epidemiologically, concomitant viral infections other than HIV are known to be associated with poor TB outcomes. In Taiwan Hepatitis C infection was associated with a higher risk of developing active TB disease [11], and in South Africa influenza/TB co-infection was associated with increased mortality [12]. A large TB vaccine trial in South Africa, which investigated correlates of TB disease risk or protection, found an association between CD8 T-cell activation

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and HCMV response [8] that was linked to an increased risk of TB disease and shorter time to diagnosis [13].

The ubiquitous herpes virus HCMV is known to cause immune activation [14], immune senescence [15], and is a significant factor in immune variation [16]. Recently, our group has reported elevated levels of HCMV IgG among TB patients compared to controls [17]. Despite this, and the findings of some early epidemiologic studies [18, 19], data linking HCMV and TB are sparse.

To investigate mycobacteria-specific antibody levels across ages, and to examine potential effects of HCMV co-infection on antibody levels, this study tested 2187 stored serum samples (of which 27 were active TB cases) from a rural Ugandan cohort for IgG responses to Ag85A, purified protein derivative (PPD), lipoarabino-mannan (LAM) and CFP10/ESAT6, along with IgM responses to Ag85A. These antigens were chosen based on availability, evidence of their potential importance in TB disease from the literature (Ag85A [9], LAM [20, 21], PPD [22]), and specificity to *M.tb* (CFP10/ESAT6 [23]). Seropositivity to HCMV was measured and existing data on HIV, BCG vaccination status, as well as demographic information was matched and investigated for associations. Responses to tetanus toxoid (TT) and total IgG levels were also investigated as control antibodies.

Materials and methods

Study area and design

The General Population Cohort (GPC) is a population-based open cohort study in rural south-western Uganda, administered by the Medical Research Council (MRC) UK in collaboration with the Uganda Virus Research Institute (UVRI) [24]. The cohort comprises a cluster of 25 neighbouring villages with approximately 20 000 residents (52% aged <13 years) from three ethnic groups, the majority (75%) being from the Baganda tribe, the main tribal group in the region. Data are collected through an annual census, questionnaire and serological survey (further details on questions included in the census can be found in a publication by Asiki *et al.* [24]). Demographic information was collected at the same time as the blood samples. Blood specimens were obtained and immediately tested for HIV-1. Samples with inconclusive results were retested. Remaining sera were stored at -80°C . All samples used for this study, except active TB cases, were collected from adults and children in GPC round 22, conducted in 2011. Active TB cases were diagnosed through positive sputum smear microscopy after passive case detection and were sampled from a range of GPC rounds between 1999 and 2014. Sera collected as

close as possible to the time of TB diagnosis were used for this study (between 5 years prior to, and 0.8 years after diagnosis of active TB diagnosis).

Sampling

Individuals were selected for inclusion in this cross-sectional study at random after stratification by age and sex. Due to anticipated high levels of HCMV seropositivity, infants under the age of 5 were oversampled. Individuals were only sampled once and siblings and parent-child pairs were not excluded. The total planned sample size was 2000, plus 10% oversampling. A target of approximately 100 individuals per year of age up to 5 years, 200 individuals aged 6–10, 11–15 and 16–20, 200 in subsequent 10-year intervals up to 60 years and 200 people aged 61 years or over. The sex ratio was approximately equal within each age group (Table 1).

Ethics

Written consent or assent in conjunction with parental/guardian consent for those younger than 18 years were obtained following Uganda National Council of Science and Technology (UNCST) guidelines before all survey procedures [24]. Written consent/assent was also obtained from participants on the use of their clinical records and stored biological samples for research purposes.

Ethical approval for the use of GPC samples for this study was obtained from the UVRI Research and Ethics Committee and from the UNCST, in addition to the London School of Hygiene & Tropical Medicine (LSHTM), London, UK.

Serology: Ag85A, PPD, LAM, CFP10/ESAT6 and TT

Antibody content was measured using ELISA. Experimenters were blind to exposure group for all samples. Testing was conducted at UVRI in Entebbe, Uganda (Ag85A IgG and PPD IgG) and LSHTM, London, UK (CFP10/ESAT6 IgG, Ag85A IgM, LAM IgG and TT IgG). Half volume ELISA plates (Fisher Scientific) were coated overnight at 4°C with sodium carbonate buffer containing 1.5 $\mu\text{g/ml}$ recombinant Ag85A protein (Aeras, USA), 1.5 $\mu\text{g/ml}$ PPD (Lot 051815KA, Aeras, USA), 0.5 $\mu\text{g/ml}$ TT (02/232, NIBSC, UK), 0.5 $\mu\text{g/ml}$ LAM (NR-14848, BEI Resources, VA, USA), 0.25 $\mu\text{g/ml}$ each CFP10 (NR-49425, BEI Resources, VA, USA) and ESAT6 (NR-14868, BEI Resources, VA, USA). Plates and reagents were brought to room temperature and the plate was washed three times with PBS, 5% Tween20 (v/v) (PBST). After blocking with PBS 5% milk (w/v) blocking

Table 1 Characteristics of study participants (HCMV seropositive individuals by age group ($n = 1988$)). Sex, HIV infection, BCG vaccination and TB case prevalence is shown for each age group

Age group (year)	<i>n</i>	Sex (percentage female (number/total))	HIV prevalence, percentage (number/total)	BCG vaccinated, percentage (number/total)	Active TB cases, percentage (number/total)
<1	75	47% (35/75)	4% (3/73)	76% (54/71)	0% (0/75)
1	83	51% (42/83)	1% (1/80)	96% (73/76)	0% (0/83)
2	82	52% (43/82)	1% (1/78)	90% (63/70)	0% (0/82)
3	87	47% (41/87)	1% (1/85)	93% (70/75)	0% (0/87)
4	100	52% (52/100)	0% (0/97)	94% (82/87)	0% (0/100)
5	106	54% (57/106)	1% (1/101)	8% (80/90)	0% (0/106)
6–10	183	47% (86/183)	1% (2/175)	90% (135/150)	0% (0/183)
11–15	192	49% (94/192)	0% (0/187)	81% (125/155)	1% (1/192)
16–20	204	51% (105/204)	1% (3/204)	69% (132/190)	0.5% (1/204)
21–30	216	56% (120/216)	9% (19/216)	70% (128/184)	2% (5/216)
31–40	238	52% (123/238)	13% (31/238)	57% (119/209)	4% (10/238)
41–50	166	45% (74/166)	15% (25/166)	52% (75/145)	4% (6/166)
51–60	109	49% (53/109)	6% (7/109)	53% (52/98)	4% (4/109)
61+	147	46% (68/147)	1% (2/147)	26% (37/144)	0% (0/147)
Total	1988	50% (993/1988)	5% (96/1956)	70% (1225/1744)	1.4% (27/1988)

buffer, duplicate 1:100 test serum, high and low PPD responder controls and plate blanks consisting of assay diluent alone with no serum were added in triplicate. Incubation for 2 h at room temperature and washing with PBST was followed by a one hour incubation with an appropriately diluted peroxidase-conjugated secondary antibody in PBST-5% (IgG, 1:500 dilution of goat anti-human IgG-HRP (04-10-20, KPL, USA); IgM, 1:10 000 dilution of goat anti-human IgM-HRP (Abcam ab97205, RRID: AB_10695942)). After washing with PBST, 50 μ l tetramethylbenzidine (TMB, BD BioSciences, USA) was added to each well. The plate was incubated for 15 min in the dark before the reaction was stopped by adding 50 μ l of 2M Sulphuric Acid (Sigma, USA) to each well. Absorbance was measured at 450 nm within 30 min to obtain optical density (OD), a surrogate marker of antibody titre.

Mean blank values were subtracted from all OD readings, and geometric means of duplicates were used to reduce the effect of skewed distribution. Median levels with interquartile ranges (IQR) are used for graphical representation of antibody levels in Figures 1 and 2.

Total IgG serology

All samples were tested for total IgG content at LSHTM. As above, half volume ELISA plates were coated overnight with mouse anti-human IgG at 0.5 μ g/ml (Abcam ab200699). IgG antibody standards (134.4–8.4 ng/ml) were prepared by diluting purified human IgG (Sigma I4506, RRID:AB_1163606) in PSBT-5% milk and test

sera were diluted 8×10^5 in PBST-5% Milk. Duplicate test samples, controls and blanks were incubated for one hour at 37 °C, and, after washing, samples incubated with peroxidase-conjugated goat anti-human Fc (Abcam ab97225, RRID: AB_10680850) diluted 1/500 with PBST-5% Milk for one hour at room temperature. Plates were developed with TMB, and sulphuric acid was added to stop the reaction. Plates were read at 450 nm and OD measurements converted into g/l by use of the standard curve on each plate.

Serology HCMV

Samples were tested for IgG antibodies against HCMV using a commercial ELISA kit (Novatec Immunodiagnostica GmbH) according to kit instructions (described in [17]).

Statistical analysis

Correlations between each pair of mycobacterial antibody OD levels were conducted using Spearman's rho. Because of the ubiquity of HCMV infection within this population [17], analysis was conducted on the HCMV-seropositive population only.

Individuals seropositive for HCMV IgG were further categorised into three groups according to the tertiles of HCMV antibody concentrations (measured by OD): low, medium and high.

Linear regression was used to determine the association of each antibody response (as continuous dependent

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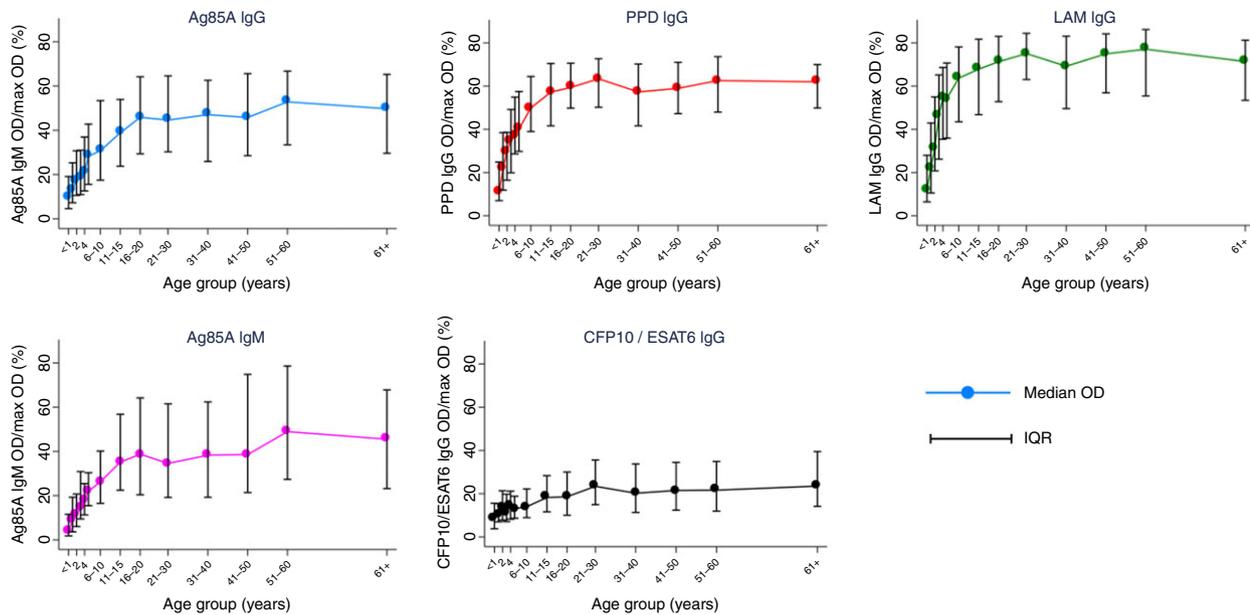


Figure 1 Median mycobacterial antibody OD by age group. Vertical lines show IQR for median values for each age group. Total $n = 1988$. OD values rescaled to the percentage of maximum for graphical representation. IQR, interquartile range, OD, optical density.

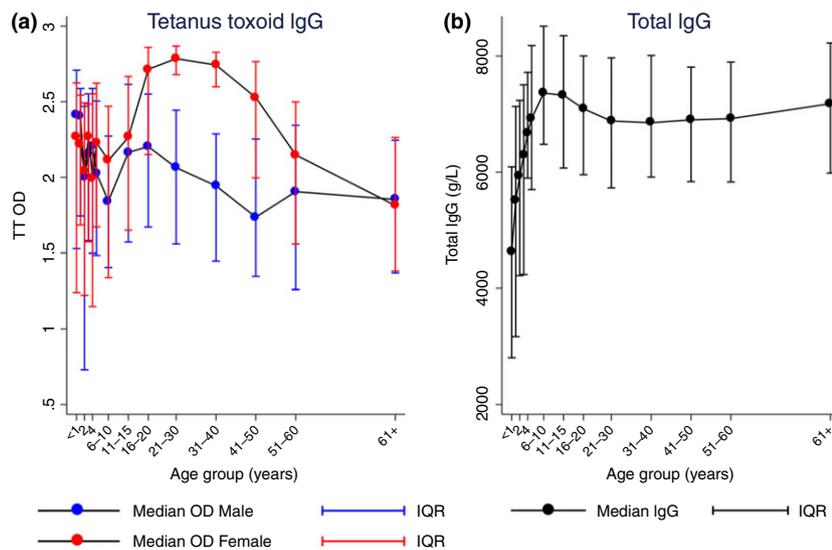


Figure 2 (a) Median OD levels for TT IgG by sex and age group. (b) Median total IgG (g/L) by age group. Vertical lines show IQR for each median data point. Total $n = 1988$. Figure 2a red lines represent median values for females, blue lines for males. Figure 2(b) shows data for males and females combined. IQR, interquartile range, OD, optical density.

variables) with HCMV IgG tertile and HIV infection (as independent variables with HCMV as a categorical variable), adjusting for age and sex. BCG vaccination status and active TB disease were also included in the regression

model, however as 89% (24/27) of active TB cases had unknown BCG status, it was not possible to include both variables in the same model. The model including TB was preferred, with the model including BCG run as a

comparison to confirm the consistency of the results. BCG vaccination was included in a separate regression analysis using the same model.

Individuals with unknown HIV status were excluded from all regression analyses and individuals with unknown BCG vaccination status were excluded from regression analyses including BCG. To account for evidence of non-linearity of antibody OD with age (likelihood ratio test $P < 0.001$ for all antibodies), a quadratic term for age was included in the regression analyses. Numbers included in each of the regression analyses are shown in Tables 2 and 3.

To account for multiple comparisons, 99% confidence intervals (CIs) are reported and a P value of 0.01 was considered to represent strong evidence to reject the null hypothesis. Due to different dynamic ranges of spectrophotometers used in Uganda and the UK, antibody OD measurements (for mycobacterial antibodies only) were rescaled for graphical representation. This was done by subtracting the minimum value within the range from each value, dividing by the range for that antibody and multiplying by 100; hence Figure 1 shows the percentage of the maximum OD. All analyses were performed on raw data using Stata version 14 (Stata Corporation, College Station, TX, USA). Anonymised participant data are available.

Results

Sera were sampled from 2189 individuals. Total IgG levels could not be determined in 15 instances, likely due to protein degradation. Of the remaining 2174 individuals, 8.6% (186/2174) were HCMV-seronegative and were excluded from analysis. The HCMV-negative population contained no TB cases and 4 HIV-positive individuals. Ninety-eight (53%) were female and the mean age was 14.6 years (range 3 months–95 years).

The remaining 1988 individuals were included in further analyses. The mean age was 23.4 years (range 30 days–100 years) and 50% were female (Table 1). Ninety-eight per cent (1956/1988) of individuals had a conclusive HIV result, and of those, 4.9% (96/1956) were HIV-positive, with the greatest proportion in the 31–50 year age group.

Twenty-seven sputum-confirmed TB cases were included in the study, of whom 63% (17/27) were female and whose mean age was 36 years (range 12–59 years). Only three TB cases had BCG vaccination information and all of those were unvaccinated. All 27 were HCMV-seropositive, with 59% (16/27) having HCMV IgG levels in the upper tertile.

Antibody responses

IgG against Ag85A, PPD, LAM, CFP10/ESAT6 and IgM against Ag85A all increased from birth to approximately 20 years of age, after which OD values reached a plateau. Figure 1 shows median OD for each of the mycobacterial antibody levels by age group. Initial levels in infants under the age of one year range from a median of 4% of maximum OD (0.12 OD) for Ag85A IgM to 12% of maximum OD (0.35 OD) for LAM IgG. Responses to the *M.tb*-specific ESAT6/CFP10 antigens did not show the same rapid increase from birth as responses to other mycobacterial antibodies. The highest 10% of responders in terms of CFP10/ESAT6, being *M.tb* specific antigens, were investigated further and there was no evidence of a difference in proportion of TB cases or HIV positive individuals compared with the lowest 10%.

Univariate analysis showed no differences in mean OD levels by sex. Analysis of correlation showed a high degree of co-linearity between all mycobacterial antibodies ($0.33 > \rho < 0.65$, all $P < 0.001$). OD ranged from 0.002 to 1.793 OD for Ag85A IgG, 0.000 to 1.930 OD for PPD IgG, 0.004 to 2.961 OD for LAM IgG, 0.005 to 2.927 OD for CFP10/ESAT6 IgG and 0.001 to 2.926 OD for Ag85A IgM.

Tetanus toxoid and total IgG levels were investigated as control antibodies (Figure 2). TT vaccination induces high levels of TT IgG and is given at birth and to pregnant females as part of the Uganda immunisation schedule [25]. TT IgG showed high levels of antibody from birth and a clear sex difference with females having higher overall IgG levels ($P < 0.001$, Figure 2a). This difference is driven by females aged between 16 and 50 years (Figure 2a). Total IgG levels increase with age from birth (median 46.2 g/l in infants less than one year of age) to a plateau after 11 years of age (median 69.4 g/l), Figure 2b. Since a univariate analysis of total IgG by sex indicated no difference, data were not separated by sex for this plot.

In a multivariable linear regression model including TB disease status, being HIV-positive was associated with a decrease in all mycobacterial antibody levels studied (magnitude ranged from mean decrease in 0.240 OD (99% CI -0.361 , -0.119) for IgG against Ag85A), to a mean decrease in 0.446 OD (99% CI -0.640 , -0.251) for IgM against Ag85A, all $P < 0.001$, Table 2). Similarly, being in the upper tertile of HCMV IgG response was associated with a decrease in Ag85A, PPD and LAM IgG levels compared to being in the middle tertile: a mean decrease in 0.067 OD (99% CI -0.122 , 0.012) for Ag85A IgG, 0.057 OD (99% CI -0.105 , -0.009) for PPD IgG and 0.107 (99% CI -0.194 , 0.019) for LAM

L. Stockdale *et al.* HIV, HCMV and mycobacterial antibody levels**Table 2** Multivariable linear regression of variables with a potential influence on mycobacterial antibody levels. Values obtained using a multivariable regression model including age, quadratic age, sex, HIV and TB status, with *P* values and 99% CI (*n* = 1956)

Variable	<i>n</i> (%)	Ag85A IgG OD		PPD IgG OD		LAM IgG OD		CFP10 ESAT 6 IgG OD		Ag85A IgM OD	
		Coeff (99% CI)	<i>P</i> value								
Sex											
Male	978/1956 (50.0%)	Baseline									
Female	978/1956 (50.0%)	-0.002 (-0.045, 0.041)	0.9	-0.002 (-0.042, 0.038)	0.91	-0.017 (-0.099, 0.064)	0.58	-0.025 (-0.083, 0.033)	0.27	-0.012 (-0.103, 0.079)	0.73
HCMV tertiles											
HCMV low	650/1956 (33.2%)	-0.037 (-0.090, 0.016)	0.07	-0.009 (-0.051, 0.033)	0.57	-0.061 (-0.151, 0.030)	0.08	-0.100 (-0.156, -0.044)	<0.001	-0.104 (-0.205, -0.004)	0.01
HCMV medium	650/1956 (33.2%)	Baseline									
HCMV high	656/1956 (33.5%)	-0.067 (-0.122, -0.012)	<0.001	-0.057 (-0.105, -0.009)	<0.001	-0.107 (-0.194, -0.019)	<0.001	-0.015 (-0.077, 0.047)	0.54	-0.058 (-0.152, 0.035)	0.11
HIV status											
Negative	1860/1956 (95.1%)	Baseline									
Positive	96/1956 (4.9%)	-0.240 (-0.361, -0.119)	<0.001	-0.310 (-0.422, -0.198)	<0.001	-0.420 (-0.586, -0.253)	<0.001	-0.266 (-0.409, -0.123)	<0.001	-0.446 (-0.640, -0.251)	<0.001
TB status											
Negative	1929/1956 (98.6%)	Baseline									
Positive	27/1956 (1.4%)	-0.101 (-0.322, 0.121)	0.24	-0.035 (-0.217, 0.147)	0.62	0.158 (-0.107, 0.424)	0.13	0.175 (-0.241, 0.590)	0.28	-0.135 (-0.609, 0.340)	0.46

Table 3 Multivariable linear regression of variables with a potential influence on total IgG and TT-specific IgG levels. Values obtained using a multivariable regression model including age, quadratic age, sex, HIV and TB status, with *P* value and 99% CI (*n* = 1956)

Variable	<i>n</i> (%)	Total IgG (g/l)		TT IgG OD	
		Coeff (99% CI)	<i>P</i> value	Coeff (99% CI)	<i>P</i> value
Sex					
Male	978/1956 (50.0%)	Baseline		Baseline	
Female	978/1956 (50.0%)	-0.326 (-2.350, 1.697)	0.68	0.272 (0.188, 0.356)	<0.001
HCMV tertiles					
HCMV low	650/1956 (33.2%)	0.318 (-2.240, 2.877)	0.75	-0.090 (-0.187, 0.008)	0.02
HCMV medium	650/1956 (33.2%)	Baseline		Baseline	
HCMV high	656/1956 (33.5%)	0.915 (-1.508, 3.338)	0.33	0.065 (-0.029, 0.159)	0.08
HIV status					
Negative	1860/1956 (95.1%)	Baseline		Baseline	
Positive	96/1956 (4.9%)	9.110 (3.735, 14.485)	<0.001	-0.172 (-0.357, 0.012)	0.02
TB status					
Negative	1929/1956 (98.6%)	Baseline		Baseline	
Positive	27/1956 (1.4%)	-5.789 (-13.298, 1.720)	0.05	-0.081 (-0.328, 0.165)	0.4

CI, confidence interval; OD, optical density.

IgG (all *P* < 0.001, Table 2). While the direction of change was the same for CFP10/ESAT6 IgG and Ag85A IgM, the mean decrease associated with being in the upper tertile of HCMV IgG response was not sufficiently large to provide good evidence of a difference based upon our significance threshold (Table 2). The association between mycobacterial antibody levels and magnitude of HCMV IgG response was not linear (Figure 3): in comparison to the middle tertile of HCMV IgG response, the lowest tertile was also associated with a decrease in mycobacterial antibody levels. A mean decrease of 0.100 OD (99% CI -0.156, -0.044) *P* < 0.001 was seen for CFP10/ESAT6 IgG and a mean decrease of 0.104 OD (99% CI -0.205, -0.004) *P* = 0.01 for Ag85A IgM. The direction of change was the same for the remaining mycobacterial antibodies (Table 2).

Being a sputum-confirmed active TB case was not associated with any differences in any mycobacterial antibody level after adjusting for age, sex, HIV and HCMV level, although given that there were only 27 cases, there was limited statistical power to detect associations (Table 2). Coefficients of the effect of HIV and HCMV for each outcome measurement were similar for a model including BCG instead of TB, and the magnitude of effect was not altered by adjusting for total IgG. Being BCG vaccinated was associated with a 0.115 OD increase in TT IgG (99% CI 0.004–0.227) *P* = 0.01.

Table 3 shows that HIV positivity was associated with an increase in total levels of IgG (9.110 g/l (99% CI 3.735, 14.485), *P* < 0.001). Being female was associated with an increase in TT of 0.272 OD (99% CI 0.188, 0.356, *P* < 0.001) but no difference in total IgG levels.

While being an active TB case was not associated with any differences in TT IgG, being a TB case was associated with a 5.8 g/l decrease (99% CI -13.298, 1.720) in total IgG however this was not significant (*P* = 0.05). The magnitude of HCMV IgG response did not affect either total IgG levels or TT IgG response (Table 3).

Discussion

In this study, we found that HIV infected individuals had decreased levels of all mycobacterial antibodies studied, with a concomitant increase in total IgG. We also found that individuals with the highest levels of HCMV IgG (indicative of highest exposure [26, 27]), had decreased levels of IgG specific for Ag85A, PPD and LAM, but had no corresponding change in total IgG levels. Previously our group has shown that TB patients have increased levels of HCMV IgG [17] and that HCMV positive infants (as measured by HCMV IFN- γ ELISPOT) were at higher risk of progressing to active TB disease [13]. HCMV infection, being linked to poor long-term health outcomes [28, 29] and large immune system subversion [16, 30], may be acting to manipulate mycobacterial antibodies which may be contributing to protective mechanisms against *M.tb* infection.

As has been seen previously [31], humoral responses against RD1 antigens ESAT6 or CFP10 (mycobacterial proteins associated specifically with *M.tb* and not NTM or BCG) did not show an association with active TB cases in this study. Due to a lack of active TB case-finding in the GPC, it is likely that some of the individuals classed as TB negative here were latently infected with

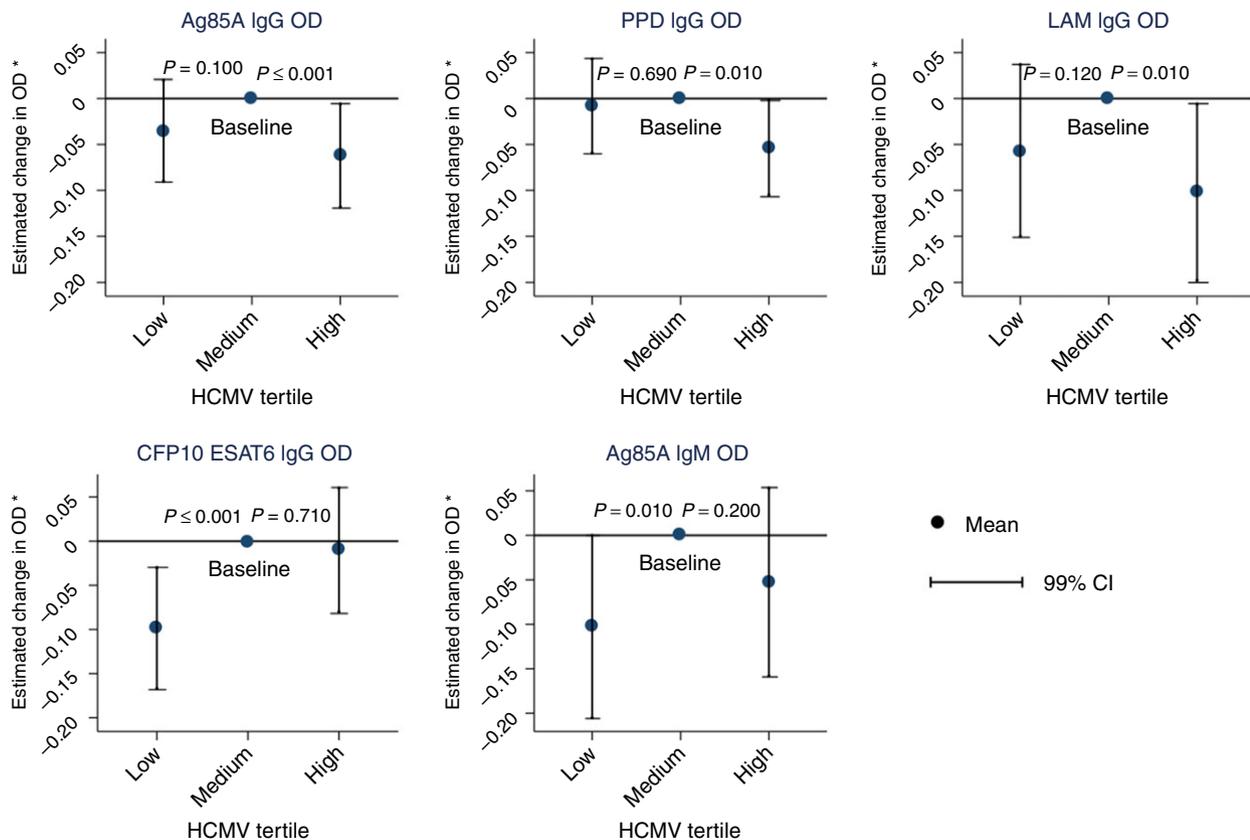


Figure 3 Adjusted mean OD change associated with HCMV tertile comparing to medium HCMV tertile as baseline. *P* values from multivariable linear regression model including age, quadratic age, sex, HIV and TB. Total $n = 1956$. Vertical lines show 99% CI for each mean OD change. CI, confidence interval, OD, optical density (*model includes, age, quadratic age, sex, HIV and TB).

M.tb based on epidemiologic data on exposure in Uganda [32, 33]. The finding that IgG responses to these *M.tb*-specific antigens increase with age, following a similar pattern as other non-*M.tb*-specific mycobacterial antibodies, suggests that while NTM may be driving high responses of acute (IgM) and sustained (IgG) mycobacterial responses, exposure to *M.tb* itself cannot be ignored.

BCG vaccine status allocation in this study was by the presence of scar, inspection of immunisation card or verbal confirmation by parent of guardian. Despite issues with these methods [34], classification was thought to be robust due to the evidence of association of TT IgG with BCG indicating that people vaccinated for one Extended Programme of Immunization (EPI) schedule vaccine are more likely to have received other vaccinations upon accessing local health centres.

The magnitude of difference in mycobacterial antibodies seen due to HIV infection was far greater than due to either active TB disease or BCG vaccination. The lower levels of mycobacterial antibodies seen in individuals

infected with HIV is accompanied by an increase in total IgG. The effect seen here points towards HIV infection having a mycobacteria-specific effect as opposed to a more general depression of all antibody levels, thereby confirming evidence seen previously in a much smaller number of patients in Italy [35]. It is known that HIV preferentially depletes TB-specific CD4 T cells [36]. Through their absence, TB-specific T cells would not provide signals to B cells in order for them to proliferate and differentiate into immunoglobulin-secreting plasma cells. As far as we are aware, the possible downstream humoral mechanism of HIV-associated TB-specific T-cell depletion has not been investigated.

Here we see a similar association between the highest levels of HCMV IgG, and decreased levels of some mycobacterial antibodies. This is not accompanied by a decrease in total IgG or TT IgG. As a human herpes virus, HCMV persists in a variety of cell types in a dormant state and is transmitted through body fluids [37]. Infection is not normally associated with symptomatic

disease; however, it poses a huge burden on the immune system, with maintenance of up to 30% of both CD4 and CD8 circulating memory T cells being specific to HCMV [38]. The virus is a significant factor in immune variation [16], immunosenescence [15] and causes immune activation [39], including non-specific polyclonal B-cell activation and proliferation [40].

In this study, we see decreased mycobacterial antibody levels at both HCMV IgG extremes among HCMV seropositive individuals. Evidence of HCMV effect on vaccine responses exists, showing both a detrimental impact of HCMV infection [41, 42] and a positive, 'adjuvanting', effect of HCMV on vaccine-induced antibody responses in humans [43]. Given reports that cellular responses to heterologous virus challenge in mice may depend on the initial viral dose of HCMV [44, 45] the lack of a linear response between HCMV IgG and mycobacterial antibody levels seen in this study may also be indicative of different effects at different HCMV exposure levels. PBMC samples were not available from this cohort for us to determine if the magnitude of HCMV IgG level had a similar impact on the mycobacteria-specific cellular immune response. However, it is likely that cellular immunity is also impacted by HCMV given that HCMV has been shown to affect both humoral and cell-mediated immune responses to vaccine antigens [46, 47].

Before the widespread use of highly active anti-retrovirals (HAART), HCMV/HIV co-infection saw HCMV as an important cause of severe non-AIDS events, including death, in HIV-infected individuals [48]. High levels of HCMV IgG, caused by repeated reactivation or reinfection events, are associated with higher all-cause mortality [49] and lower CD4 cell count, with accompanying worse response to anti-retrovirals [50]. Despite literature describing HCMV-induced B-cell activation [40, 51, 52], in this study we saw no relationship between HCMV exposure and increased levels of total IgG. We do however see a trend towards decreased mycobacterial antibody response in individuals with very high HCMV IgG responses after adjusting for HIV infection. Considering the risk of developing active TB disease increases over 20 times in individuals with HIV [53], it is likely that any added effect of HCMV would be masked. The mycobacterial antibody-specific decrease seen with both HIV infection and, independently, in the highest tertile of HCMV exposure, may be indicative of a functional, protective role of mycobacterial-specific antibodies which are independently decreased by HIV and high levels of exposure to HCMV.

In summary, both HIV infection and high levels of HCMV IgG were both associated with decreases in mycobacterial antibody levels. Given this novel finding of

effect of HCMV exposure on mycobacterial antibody levels, investigation of magnitude of HCMV may be important in future TB clinical trials to understand the immune environment, independently of HIV infection status. In addition to quantification of antibody levels, we believe that it will be important to measure HCMV effect on mycobacteria-specific cellular responses. A more nuanced understanding of the quality, as well as the quantity, of the mycobacterial antibodies elicited, in terms of antibody class, subtype and avidity may help to shed light upon a potential mechanism by which they might confer protection to host cells.

Due to insufficient volumes of sera, we were unable to investigate HCMV viral load in serum samples to ascertain whether active infection at point of blood draw was associated with any of the measurements taken for this study. Despite this, we believe that the use of HCMV-specific IgG is a robust a measure of cumulative exposure. As previously mentioned, active TB case finding is not carried out within the GPC and therefore we cannot be sure that some individuals analysed as 'non-TB' are not latently infected. PBMC samples were not available from this cohort for us to determine if HCMV had a similar impact on the mycobacterial cellular immune response.

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