The protective role of secretory IgA and IgG against *Mycobacterium tuberculosis* infection

Rubiyat E Islam

Supervisors:
Fekadu Abebe, (PhD)
Dominique A. Caugant, (PhD)
Liya Wassie, (PhD)

Thesis submitted as a part of the Master of Philosophy Degree in International Community Health

Department of Community Medicine
Institute of Health and Society
Faculty of Medicine
University of Oslo

November 2022

Abstract:

Background: Tuberculosis (TB) is a global disease which infects approximately one-quarter of the world's population. However, only 5-10% develop clinical TB, suggesting the presence of immune protection. To date, the protective role of antibodies in respiratory mucosal lining is not well documented. Understanding the role of mucosal immunity against TB is important to replace currently used Bacillus Calmette-Guerin (BCG) and develop a new vaccine. The project aims to compare the induction and protective role of secretory IgA and IgG in serum and saliva against the *Mycobacterium tuberculosis* (*Mtb)* antigens lipoarabinomannon (LAM) and heparin-binding hemagglutinin adhesin (HBHA) among pulmonary TB patients (PTB), household contracts (HHC) to TB patients, and community controls (CC).

Methods: In this cross-sectional study, we included a total of 90 participants within three study groups (PTB, HHC, CC), each with 30 participants. Structured interviews with close-ended questionnaires and sample collection were conducted in Addis Ababa, Ethiopia. Sera and saliva samples were collected and assayed by enzyme-linked immunosorbent assay (ELISA). Sputum smear microscopy and gene- Xpert test were used to identify pulmonary TB patients and QuantiFERON-TB GOLD in-tube (QFT-GFT) was done to screen TB infection. Data analyses were performed using IBM SPSS (version 28) and GraphPad Prism version 8.0.0 for windows.

Results: Our results showed that IgA responses to HBHA in HHC were significantly higher than PTB patients in both saliva and serum ($P<0.05$). QFT-negative groups had higher anti-HBHA IgA responses than PTB patients ($P<0.05$), and HBHA-stimulated IgA responses were higher in HHC compared to HBHA-unstimulated IgA levels in HHC and CC in serum ($P<0.05$). In comparison to PTB patients, both HHC and CC as well as QFT-positive and QFT-negative had higher IgA responses to HBHA ($P< 0.05$, $P<0.01$, $P< 0.05$, $P<0.01$, respectively) and LAM ($P< 0.05$, $P<0.01$, $P<0.01$, $P<0.01$, respectively). Anti-LAM and anti-HBHA IgA levels were significantly higher in the saliva of the study participants compared to their levels in serum ($P<0.0001$).

Conclusion: The findings of this study suggest the presence of protective immunity and role of IgA and IgG in preventing the development of active and latent TB. In addition, for the first time, our study reports the protective role of secretory IgA (mucosal immunity) against HBHA in saliva, which may have significant implications for future TB vaccine development.

Keywords: Tuberculosis, IgA, IgG, antibody, mucosal immunity, antigen, saliva, serum.

Acknowledgement:

First and foremost, praises and thanks to the Almighty, the God, for His showers of blessings throughout my research work to complete the thesis successfully.

I would like to express my sincere gratitude to Prof. Fekadu Abebe for offering his supervision, guidance from the initial step in thesis, which helped me to develop an understanding of the subject. Thank you for inspiring me to complete this research project. I have benefited greatly from the extraordinary experiences Prof. Fekadu arranged for me and for providing the opportunities for me to grow professionally. It was an honor to learn from you.

I am extremely grateful to Prof. Dominique A. Caugant for her mentorship and for sharing her expertise as well as sincere, unwavering support for the thesis. I appreciate your invaluable knowledge and contribution in aiding the completion of the thesis.

I would like to offer my special thanks to Dr. Liya Wassie for her professional advice, expertise, and positive criticism which was influential in shaping my research methods and enabled me to improve and re-analyze the problem statement. Your ideas and insights made it much easier to proceed in the right direction and I am grateful for that.

Further, importantly, sense of respect goes to Armauer Hansen Research Institute (AHRI). I am grateful for the generous support that I have received from AHRI while my stay in Ethiopia. I would like to thank Yonas Abebe for his assistance in the participants recruitment process and field co-ordination in Addis Ababa. I would also like to thank my lab mates and colleagues, Meaze Zewde and Danial Mussa, for those invaluable lessons and the time we spend together in the lab. In addition, I would like to show my gratitude to Mekdi and Medina for the kind help and support that have made my time wonderful in Ethiopia.

My appreciation also goes to Terese Eriksen and Birthe Neset for their encouragement and support all through my master's program. I would like to thank University of Oslo for providing the financial support for the thesis. Furthermore, I am grateful to share the classroom with a unique and diverse set of people from all over the world.

Finally, I want to express my gratitude to my family, close friends, and loved ones for their unconditional love and support.

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List of Acronyms

Acr	Alpha crystallin
ADCC	Antibody-dependent cell cytotoxicity
AIDS	Acquired immunodeficiency disease
AM	Arabinomannan
APC	Antigen presenting cell
BCG	Bacille Calmette-Guerin
BAL	Bronchoalveolar lavage
CC	Community control
CD4	Cluster differentiation 4
CFP-10	Culture filtrate protein 10
CFU	Colony forming unit
DC	Dendritic cells
DOTS	Directly observed treatment short course
ELISA	Enzyme-linked immunosorbent assay
ESAT-6	Early secreted antigen target 6
FcR	Fragment crystallizable receptor
HBHA	Heparin binding hemagglutinin
HHC	Household contacts
HIV	Human immunodeficiency virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IGRA	Interferon-gamma release assay
IL	Interleukin
IFN-γ	Interferon-gamma

LAM	Lipoarabinomannan
LPS	Lipopolysaccharide
LTBI	Latent TB infection
MALT	Mucosal-associated lymphoid tissue
mAb	Monoclonal antibody
MDR-TB	Multidrug resistant tuberculosis
Mtb	Mycobacterium tuberculosis
NK	Natural killer
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PIgR	Polymeric immunoglobulin receptor
PHA	Plant phaseolus vulgaris agglutinin
QFT-GIT	QuantiFERON TB Gold In Tube
RR-TB	Rifampicin resistant TB
RT	Room temperature
SIgA	Secretory IgA
SDG	Sustainable Development Goals
TB	Tuberculosis
TNF-α	Tumor necrosis factor-alpha
WHO	World Health Organization

1. Background:

1.1 Tuberculosis:

Tuberculosis (TB) is a bacterial infectious disease caused by *Mycobacterium tuberculosis (Mtb)*. In 2020, TB claimed the lives of 1.5 million people, making it the leading infectious disease after Covid-19 (World Health Organization, 2021). Despite the End TB Strategy and Bacille Calmette-Guerin (BCG) vaccination, a third of world's population continues to get infected with TB and it has become a global burden for the low to middle income countries. Following the Covid-19 pandemic, the interruption of TB services has escalated the infection rate and TB related fatalities, posing a threat to lives and livelihood of people around the globe (World Health Organization, 2021). The primary obstacles to the control of the disease are the lack of adequate tools for TB prevention and diagnosis, human immunodeficiency virus (HIV) coinfection, and new emerging drug- resistant TB (Petersen, 2022). On average, 45% of HIV-negative people with TB on average and approximately all HIV-positive people with TB will die without proper preventive TB treatment, indicating the urgency for a better vaccine and preventive measures (World Health Organization, 2021).

1.2 *Mycobacterium tuberculosis (Mtb)*:

Mtb, is a potent human pathogen and the causative agent of TB, belongs to the order of *Actinomycetales (*Deretic & Fratti, 1999; Chuquimia, 2011) and is the member of specie *Mycobacterium tuberculosis* complex (MTBC) (Delogu et al., 2013). This complex is comprised of *Mycobacterium tuberculosis (Mtb); Mycobacterium africanum*, responsible for pulmonary TB in humans in specific regions of Africa; *Mycobacterium bovis*, *Mycobacterium caprae*, and *Mycobacterium pinnipeddii*, which causes TB in domestic and wild animals; and *Mycobacterium microti*, that causes TB in small rodents, such as voles (Delogu et al., 2013). Humans are the sole reservoir of *Mtb* (Bennett et al., 2019). This bacillus spreads from person to person through respiratory droplets which are emitted by individuals with active TB and inhaled by the contacts. Active TB patients can spread the disease through close contact to 5-15 other people over the course of a year (World Health Organization, 2021). *Mtb* is an aerobic, rod-shaped, non-motile intracellular bacterium which takes 15-20 hours to replicate (Bennett et al., 2019). The organism can be recognized by its acid-fastness (Bennett et al.,

2019). The tubercle bacillus is unique since it has an intricate cell envelope which is further composed of a cell wall and cytoplasmic membrane. This envelope is a key component of the pathogenicity of *Mtb*, promotes drug resistance, and facilitates the viability of *Mtb* in adverse conditions (Alvarez-Corrales, 2014). The cell wall comprises long-chain fatty acids, glycolipids, peptidoglycans, and proteins (Cole et al., 1998). Long fatty acid chains are micolic acids, which are characterized by cyclopropane rings and double bonds, and through an arabinogalactan polymer made of arabinose and galactose subunits, they connect to peptidoglycans (Alvarez-Corrales, 2014).

2. Literature review

2.1 Epidemiology of TB

2.1.1 Global and regional TB report:

A systematic analysis of the TB incidence globally and the effect of Covid-19 on TB control strategies were presented in the World Health Organization (WHO) global tuberculosis (TB) report 2021. The report stated that approximately 10.0 million people became ill with TB globally in 2020, equivalent to 127 cases per 100,000 populations (World Health Organization, 2021). However, it was likely that about 5.8 million cases were not notified, which have caused an 18% decline in the number of individuals reported to have had a TB diagnosis in 2020 (World Health Organization, 2021). Around 16 nations accounted for much of this decline, with Asia (particularly China, India, Indonesia, and the Philippines) experiencing the biggest drop in case reporting. There were significant setbacks in health care and Covid-19 outbreaks in each of these countries (Pai et al., 2022).

TB mortality has increased worldwide for the first time in over 10 years. WHO reported approximately 1.5 million TB deaths among HIV-negative people and estimated 214000 deaths among HIV positive people in 2020, compared to the mortality rate in 2019, which was 1.2 million and 209,000 fatalities among HIV negative and positive cases, respectively (World Health Organization, 2021). This is due to restricted access to TB-related care and facilities during the concurrent Covid-19 pandemic. The highest TB mortality rate was mostly documented in 30 high TB burden countries, including Angola, Congo, Bangladesh, China, and Brazil (Bagcchi, 2021). Other detrimental pandemic-related impacts include a 15% drop in the number of patients receiving treatment for drug resistant TB, with a subsequent 21%

fall in those receiving therapeutic treatment for TB infection. This leads to a decrease in global TB expenditure from 2019 to 2020 (from $5.8 billion to $5.3 billion) (World Health Organization, 2021).

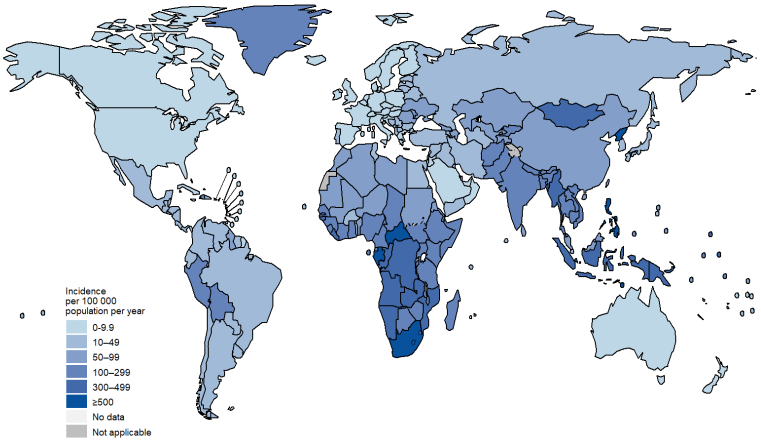


Figure 1:Estimated TB incidence rate 2020, taken from (World Health Organization, 2022)

Africa is one of the most TB prevalent regions due to high TB/HIV co-morbidity, which plays a key role in TB outbreaks and related mortality (Teferi et al., 2021). Around 2.5 million people become ill in the African region in 2019, which is the 25% of the universal TB burden (World Health Organization, 2022). In 2020, the WHO African Region had the highest percentage of TB cases with HIV coinfection, surpassing 50% in some areas of southern Africa. Around 84% of TB deaths among HIV-negative people and 85% of all TB among HIV-positive and HIV-negative individuals were recorded in the WHO Africa and South-East Asia Region (World Health Organization, 2022).

Ethiopia, located in the horn of Africa, is one of the high TB burden countries globally (Haileamlak, 2021). Among the 30 most severe tuberculosis-endemic countries worldwide, Ethiopia ranked number 10 with an estimated 164 TB cases per 100,000 people (Kahase et al., 2020). In 2020, HIV associated TB incidence was 8.6 per 100,000 and the fatality rate among HIV-positive individuals was 2.2 per 100,000 population (Seyoum et al., 2022). The prevalence of rifampicin resistant TB (RR-TB) and multidrug resistant TB (MDR- TB) in new cases were 1.1%, 1.03%, and 7.5%, 6.52% among those that had already received treatment in 2019 (Belachew et al., 2022). TB is among the ten most prevalent public health problems in Ethiopia affecting both urban and rural populations with varying magnitudes. Roughly 62% TB cases were confirmed by clinical diagnosis, with a national case detection rate of

approximately 66% (Law et al., 2020). The current WHO global TB report 2021 indicates the treatment success rate to be 90% in new and relapse cases (World Health Organization, 2021). Ethiopia is one of the seven high TB burden countries which attained the End TB Strategy's first benchmark- a 20% decline in TB incidence between 2015 and 2020. Nevertheless, TB continues to be a major health concern in Ethiopia due to HIV coinfection and socioeconomic determinants, namely poverty and inequality (Chilot et al., 2021).

2.1.2 TB control measures:

In the past 20 years, several strategies have been implemented and adapted to control global TB. Back in 1993, recognizing the major outbreaks of TB in most of the developing countries, WHO took an unprecedented step and declared TB as a global emergency (Sulis et a., 2014). In 1995, the Directly Observed Therapy, Short Course (DOTS) was first introduced by WHO (Lienhardt et al., 2012). Although DOTS strategy contributed to make a significant progress, still the program was not adequate to reach the international goal of eradicating TB mortality and prevalence by 2015 (Lienhardt et al., 2012). Following 10 years of DOTS, the new Stop TB strategy was launched by WHO in 2006 (Haileamlak, 2021). The End TB Strategy is more universal approach that considers the unprivileged population, MDR/RR-TB, HIV-TB coinfection, reform of health system, and research (Lienhardt et al., 2012). Furthermore, target 3.3 of the UN Sustainable Development Goals (SDGs) aims to eradicate TB by the end of 2030 (Merk et al., 2019). Three major sub-targets were further established by the End TB Strategy to assess the progress towards the SDGs. These sub-targets included that the TB incidence should be 80% lower in 2030 in comparison to 2015, TB mortality should be 90% less, and no family should incur fatal losses because of TB (Merk et al., 2019).

The End TB Strategy has not made satisfactory progress toward the objectives put forth as of now. Nine nations with the highest rate of TB cases - experienced a sharp fall (18%) in diagnosis and treatment of TB infection, ranging from 16-41% in 2020, one year after Covid-19 pandemic rocked the world (Malik et al., 2022). Additionally, a substantial proportion of undiagnosed TB cases and persistently low case detection and treatment success rates in countries with high TB burdens render the DOTS passive case finding approach problematic (Abayneh et al., 2020). Thus, we require improved diagnostic tools, an affordable and shorter drug regimen, and a new effective vaccine will be required to meet the targets of SDG and End TB by 2030 and 2035, respectively.

2.1.3 Shortcomings of current TB Vaccine:

The sole solution of regulating and halting the spread of infectious disease is the widespread use of efficient vaccination (Handzel, 2013). Bacillus Calmette-Guerin (BCG), an attenuated strain of *M. bovis*, developed a century ago and is the single currently authorized anti-TB vaccine (Sia & Rengarajan, 2019). With over 4 billion vaccinations worldwide and around 100 million neonatal vaccinations yearly, BCG is among the most used vaccines in the world (Moorlag et al., 2019), because of its affordability, consistency, and reliability (Ernst, 2018). Since the early 1900s, BCG has been providing immune protection against disseminated TB in infants, in addition to TB meningitis and miliary TB (Horwitz & Harth, 2003). However, the potency of BCG in hindering pulmonary TB in adults is questionable and BCG vaccination could no longer protect large adult population from emerging new infections with resistant organisms, notably MDR and extreme drug resistant (XDR) strains (Horwitz & Harth, 2003). The preventive effectiveness of BCG in adults varies from 0% in South India to 80% in the UK (Kaufmann, 2000). However, a meta-analysis of accessible vaccination data has reported a theoretical success rate of 50%, suggesting that BCG could have only precluded 5% of all vaccine-preventable fatalities attributable to TB (Kaufmann, 2000). The inefficacy of BCG has been linked to numerous variables, such as the BCG strain, route of administration, dosage, the prevalence of nontuberculous mycobacteria (NTM), host genetics background, geography, climate, as well as co-infections. Regardless of the outcomes, BCG has not been beneficial to reduce the prevalence of TB globally (Ernst, 2018).

A novel vaccine strategy should optimally be designed to stimulate the protective immune response in hosts, to minimize tissue injury, promptly obliterate bacterial growth, and prevent the progression of the disease (Delogu & Fadda, 2009). However, development of effective vaccine is impeded by insufficient knowledge regarding immune protection (Huang & Russell, 2017). Since the 1930s, several randomized controlled trials have raised questions about the efficacy of BCG because the mechanism of immune protection of BCG is still ambiguous and the role of immune marker, for instance, IFN-γ produced by CD4+ T cells in immune protection of BCG is debatable (Abebe, 2012). One of the biggest trials took place in Chennai, India reported that BCG provides less immune protection for adults and adolescents, and they are more likely to get infected than their placebo groups (Abebe, 2012). Until a mass vaccination program combined with BCG was implemented in Africa, there had been no clinical trial conducted on the continent (Abebe, 2012). There is still a substantial research gap, and this is high time to consider the gap still existing regionally and globally. Therefore,

potent vaccine developing strategies have eventually become an international research priority.

2.2 Immunopathogenesis of tuberculosis

2.2.1 Pathogenesis of *M. tuberculosis*:

Mtb establishes a chronic infection when minute aerosol particles harbouring the bacteria are landed in the lower lung of a new host from a tuberculous individual (Cambier et al., 2014). A complex and diverse immune response take places during the interaction between the host and *Mtb,* leading to active disease, latent infection or the pathogen being completely eradicated (De Martino et al., 2019). When *Mtb* enters in the pulmonary alveoli, the tubercle bacilli recruit infected macrophages and the bacteria are transported to deeper tissues by passing through the lung epithelium (Cambier et al., 2014). Dendritic cells (DCs) are known to ingest and contribute to transporting the bacteria from the site of infection to the surrounding lymph nodes. Additionally, *Mtb* has been shown to infect alveolar epithelial cells and other adjacent cells in the respiratory tract (Chuquimia Flores, 2011). Following invasion, *Mtb* arrests the activation of macrophages and develops a high defense mechanism. Through a range of immune evasion techniques, such as suppression of phago-lysosome fusion, detoxification of nitrogen and oxygen radicals, and latency, *Mtb* exploits the infected cells to escape recognition and eradication (Mayer-Barber & Barber, 2015). A key component of protection against *Mtb* is the conversion of phagosomes that contain bacteria into acidulated, antimicrobial compartments. In this context, adaptive immune cells like CD4 and CD8 T-cells assist in antimycobacterial defense by producing interferon-gamma (IFN-γ), and other cytokines and chemokines, which can stimulate infected myeloid cells and suppress bacterial growth (Sia & Rengarajan, 2019). Furthermore, insufficient cell-intrinsic response to *Mtb* or the proliferation of bacilli in adequate quantities within alveolar macrophages result in the rupture of infected cells. Infected macrophages' cell death pathways and the discharge of the bacteria from the infected cells both contribute significantly to the dissemination of the *Mtb* infection (Mayer-Barber & Barber, 2015).

Cell-mediated immunity appears 2-6 weeks after infection, causing the migration of mononuclear cells from the nearby blood cells, fibroblast, and activated macrophages to the site of primary infection, leading to form hallmark lesion called granuloma (Chuquimia Flores, 2011). Granuloma is a pathologically defining trait of host immune response against

Mtb, consisting of infected macrophages in the center enclosed by foamy giant cells and lymphocytes at the periphery (Flynn et al., 2011; Chaurasiya, 2018). Constraining *Mtb* at the initial site of infection by an internal cellular layer and an external fibrotic layer restricts the bacteria from disseminating throughout the host and provides an immunological environment where intermodulation of *Mtb* (active or dormant) and the host immune response occurs (Ulrichs & Kaufmann, 2006). Despite such crucial concentrated immune response, there is a constant risk of developing an active TB disease (Chaurasiya, 2018). In active TB cases, three distinct forms of granuloma exist side by side, commencing with solid granuloma which eventually transform into centrally necrotic granuloma because of *Mtb* becoming a metabolically active, rapidly replicating pathogen. When necrotic granulomas liquefy, caseous granulomas and cavities with tissue destruction emerge (Dorhoi & Kaufmann, 2014, June). Tissue damage with necrosis subsequently form fibrosis, which is the final safeguard of host defense mechanism. It has been reported that granuloma maturation in animal models is bacteriostatic, not eradication (Ramakrishnan, 2012). Studies also speculate that, this host immune response can prevent microbial growth, limit the spread of infection, and result in a latent TB in 90-95% of instances (Delogu & Fadda, 2009). However, 5-10% of those exposed do not eliminate the bacteria, and the host immune response also causes significant tissue damage and necrosis, which are the idiosyncrasies of disease progression in immune-compromised individuals (Delogu & Fadda, 2009).

2.2.2 Immunity against *Mtb:*

Immune response to *Mtb* infection is multifactorial and reflects a variety of host-pathogen interactions with varying results, from early clearance to dissemination of TB. Following aerosol transmission, *Mtb* encounters the antigen presenting cells (APC) in the lung including alveolar macrophages and DC, and they help in maintaining tissue homeostasis, phagocytosis of bacteria, initiation of antimicrobial pathway and local immune responses, as well as resolution of inflammation (Sia et al., 2015). During the first counter, macrophages and DC recognize pathogen associated molecular patterns (PAMPS) of *Mtb* (glycolipids, lipoproteins, and carbohydrates) by a group of germ-line encoded receptors known as pattern recognition receptors (PRRs) (Liu et al., 2017; Kleinnijenhuis et al., 2011). These host receptors include Toll-like receptors (TLCs), complement receptors, nucleotide-binding oligomerization domain- (NOD-) like receptors (NLRs), mannose receptors, and the DC-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Lerner et al., 2015). The recognition receptor molecules identify a variety of invading pathogens and trigger an immune response

to prevent their entry and neutralize their harmful effects, such as tissue damage (Dwivedy & Aich, 2011). Other innate immune cells such as neutrophils, natural kill (NK) cells also play important role in immune responses against *Mtb.* Neutrophils serve as an early line of defense against *Mtb* infection by secreting antimicrobial molecules and inflammatory mediators (Sia & Rengarajan, 2019). In addition, NK cells are the innate lymphocytes that exhibit robust cytolytic functions and can suppress *Mtb* replication through the secretion of chemokines and cytokines, such as IL-12, TNF-α, IL-22, and IFN-γ (Sia et al., 2015).

Adaptive immunity involves both T and B lymphocytes together with their effector molecules. B cells change into plasmocytes and produce antibodies (Cano & Lopera, 2013). Antigen presentation by DC into lymph nodes triggers T cell activation and differentiation into effector T cells (Cooper, 2009; De Martino et al., 2019). $CD4^+$ T cells are generally considered to be the key elements of acquired immunity to *Mtb* infection. Mycobacterial peptide antigens recognized by CD4+ T lymphocytes are degraded in phagolysosomal compartments and bonded with major histocompatibility complex (MHC)- class II molecules (Tjärnlund, 2005). In contrast, $CD8^+$ T cells identify bacterial peptides and lipids via MHC-class I molecules, leading to a cytotoxic response against the pathogens. T cell activation, through the recognition of these antigens in the early stages, generates Th1 proinflammatory cytokines including IFN-γ, IL-2, and TNF-α, which are vital for the cell-mediated immunity against *Mtb* (Alvarez-Corrales, 2014). IFN-γ is a key pro-inflammatory cytokine, and when combined with TNF-α, it stimulates macrophages, allowing for further microbicidal activity and antigen presentation (Chauhan et al., 2013). Moreover, IFN-γ stimulates nitric oxide synthase (NOS) to produce nitric oxide (NO), which aids in the elimination of intracellular bacteria (Alvarez-Corrales, 2014). Knockout mice model with defective IFN-γ gene suffered from severe *Mtb* infection (De Martino et al., 2019) and in individuals with mutations in genes involved in the IFN-γ and IL-12 pathways, supporting the hypothesis that IFN-γ is critical for control of *Mtb* infection (Chuquimia Flores, 2011).

2.3 Mucosal immunity and correlation of protection:

2.3.1 Mucosal Immunity:

The mucosal surfaces serve as the first line of defense against the entry of bacteria and viruses. A combination of non-specific and specialized mechanisms protects mucosal

membranes from colonization, potential penetration, and invasion by microorganisms (Tjärnlund, 2005). Immune system associated with mucosal surfaces composed of anatomically and physiologically dissimilar compartments, which are endowed with decontamination mechanism to provide protection at respiratory, oral, ocular, nasopharyngeal, gastrointestinal, and genital mucosa, and mammary glands (Mestecky et al., 2015). The mucosal immune system is complex and highly specialized, covering a surface area of 200-300 m^2, that has developed mechanical and chemical mechanism to differentiate among harmless antigens, commensal microorganism, and dangerous pathogens (Tlaskalová-Hogenová et al., 2002). This immune system is distinct in that it contains lymphocytes that are not found in the bloodstream and have a unique immunological response due to the presence of the secretory IgA (Dwivedy & Aich, 2011).

Mucosal immune systems can be classified into inductive and effector sites. The inductive sites are collectively known as mucosa-associated lymphoid tissue (MALT) where active immune responses take place. MALT consists of epithelial cells and differentiates microfold (M) cells, which assess foreign particles in the lumen and transport them to DC on the basal side (Stylianou et al., 2019). DC collect pathogen-specific antigens and process them prior to migrating back to the MALT or lymph nodes to initiate an immune response by T & B cells against that pathogen (Stylianou et al., 2019). MALT also acts as reservoir for memory B and T cells which travel to effector sites through the lymphatic system in the common mucosal immune system. On the contrary, mucosal effector sites encompass antigen-specific mucosal effector cells such as IgA-producing plasma cells, and memory B and T cells (McGhee & Fujihashi, 2012).

2.3.2 Importance of mucosal immunity in TB:

Mtb enters the body through the lungs. Thus, the mucosal immune system is responsible for providing protective mechanisms against *Mtb* invasion and is tightly regulated to permit immunogenic tolerance against the entry as well as contains adaptive immune regulation and provide intrinsic defence. (Neish, 2014; Janeway et al., 2001).

Following *Mtb* exposure, clinical phenotypes differ from complete pathogen clearance to immunologically confined latent infection to active TB illness. Early clearance of *Mtb* bacilli prior to T-cell sensitization in exposed individuals who appear to be resistant to *Mtb* infection may be due to innate immune systems within the lung mucosa. Overall, about 5–10% of

immunocompetent patients with suspected latent *Mtb* infection (LTBI) acquire TB illness during their lifespan, with the remaining majority achieving immunological equilibrium (Morrison & McShane, 2021). Humans produce abundant amount of IgA and IgG at the mucosal site. In contrast, like IgG, systemic IgA involves in initiating inflammatory responses together with antibody-dependent cytotoxic clearance, endocytosis, phagocytosis, superoxide radical production, cytokines, inflammatory mediators, and complement activation (Legesse et al., 2013). Furthermore, studies have shown that the IgA1 isoform of a monoclonal antibody was more protective than IgG1 isoform of same monoclonal antibody in lung epithelial cells (Li & Javid, 2018). The relevant lung epithelial cells exhibited neonatal Fc receptors, which only bind IgG and not IgA. Thus, epithelial cell binding and suppression of *Mtb* phagocytosis may be the reason for the protective action of IgA (Li & Javid, 2018). In neonates, higher levels of Ag85A-specific IgG have been correlated with a lower possibility of developing active TB disease (Pinpathomrat et al., 2021). IgA mutant mice exhibited increased bacterial colony forming unit (CFU) counts in the lungs than control mice following *Mtb* challenge (Pinpathomrat et al., 2021). Hence, it is evident that further investigation of immune responses in the lung is of particular importance and mucosally induced antibodies may be crucial for *Mtb* protection (Pinpathomrat et al., 2021).

2.3.3 Antibody-mediated protection against *Mtb* infection:

The protective mechanism of antibodies (Abs) in TB is believed to be heterogenous and mostly reliant on the antigen specificity, isotypes, site of secretion or route of delivery, if used as immunotherapy (Tran et al., 2019). Immunity appears to be relatively more complicated than neutralizing bacteria and cell-mediated actions are required as well (Li & Javid, 2018). Antibodies likely play a role in preventing *Mtb* infection during expectoration and following reinfection in host cells, which leads to dissemination of bacteria into the extracellular space (Hermann & King, 2021). In individuals with pleural TB, activated *Mtb*-specific B cells may secrete immunoglobulin which binds with extracellular bacilli. Interaction of Abs with free *Mtb* antigens or the bacillus itself leads to the activation of fragment crystallizable receptor (FcR)-mediated macrophages (Jacobs et al., 2016). Additionally, Abs against *Mtb* may also have direct microbicidal and neutralizing functions independent of the Fc-receptor by blocking the uptake of bacteria into cells (Li & Javid, 2018) or by inhibiting antigen stimulation by shifting *Mtb* towards the direction of bactericidal receptors than mannose receptors. These may result in increased phagocytosis, phago-lysosomal fusion and reduced intercellular longevity, according to some studies (Tran et al., 2019). It is conceivable that

Abs may enhance the maturation of DC and improve the efficiency of MHC class I restricted presentation of antigenic peptides to T cells (Tran et al., 2019). Another possible mechanism is that antibody specific for *Mtb* may accelerate the elimination of *Mtb* infected cells by NK cell-mediated antibody-dependent cell cytotoxicity (ADCC) (Li & Javid, 2018). Naturally induced anti-*Mtb* IgM may be able to opsonize and neutralize secreted toxin, and *Mtb*-specific IgG may accelerate the reduction of mycobacterial repository in tissues through CD16-mediated ADCC (Rao et al., 2015). Similarly, IgG have been reported to boost the generation of *Mtb*-specific IFN-γ by CD4+ and CD8+ T cells illustrating potential Abs mediated anti-TB immunity (Tran et al., 2019).

2.3.4 Antigenic targets of protective antibodies during *Mtb* infection:

Progressive antibody response to *Mtb* infection is reflecting the varying availability of antigens along the infection's spectrum in humans (Hermann & King, 2021). Antigens of *Mtb*, identified in the infection site that have been expressed in the lungs of susceptible mice, were able to excite both conventional and unconventional T cells from latently infected patients, including T cells producing cytokines other than IFN-γ (Zhu et al. 2018). Microarray studies have also demonstrated that *Mtb* infection induce the production of antibodies to a variety of these antigens (Jacobs et al. 2022). The *Mtb* genome encodes over 4000 proteins (Yang et al., 2019), and yet only a small portion of them have been investigated. Antigen based series of *Mtb* challenge studies in mice examined the protective effects of monoclonal antibodies (mAbs) against three well-known *Mtb* antigens, including heparin-binding hemagglutinin (HBHA), alpha-crystallin and arabinomannan (AM), lipoarabinomannan (LAM) (Hermann & King, 2021). LAM is a glycolipid component of the envelop of all mycobacterial species and is the fundamental carbohydrate antigen of the *Mtb* contributing around 15% of the bacterial weight (Correia-Neves et al., 2019). It serves as immune-modulating virulence factor which is associated with the pathogenesis of *Mtb* and is imperative for the entry and survival inside the cells (Correia-Neves et al., 2019). LAM is composed of a mannan "core" which is decorated by a single branched arabinan chain (Correia-Neves et al., 2019). LAM with mannosyl caps (ManLAM) impedes DC maturation and phagocytic activity, initiates cytokine release by dendritic cells and macrophages and modulates the suppression of adaptive immune responses against *Mtb* by binding to DC-specific intercellular adhesion molecule 3 grabbing non-integrin and dectin-2 receptors on dendritic cells and macrophages (Druszczynska et al., 2017). Owing to specific immunogenic characteristic and heat stability, LAM is now considered as a potential diagnostic marker to

identify infected individuals (Druszczynska et al., 2017). Recent studies have revealed that LAM-specific T cells play a crucial role in infection and support the differentiation of LAM-specific B cells into plasma cells. Additionally, in murine model of mycobacterial infection, neutralizing antibodies to LAM or its component AM may contribute to protective host immune response (Correia-Neves et al., 2019). Glatman-Freedman, A. (2003) suggested the LAM specific serum antibodies were associated with *Mtb* induced classical complement activation and responses of mAbs to the carbohydrate portion of LAM and AM, indicate that polysaccharide epitopes may have an assertive role in protective immunity.

Likewise, HBHA is a lectin like factor retrieved from *Mtb* cell wall that helps in agglutination of erythrocytes (Parra et al., 2004). It is a 28 KDa surface expressed adhesive protein which helps in initiating the mycobacterial attachment to epithelial cell wall (Delogu et al., 2006), and is highly immunogenic in nature (Teimourpour et al., 2017). HBHA-deficient *Mtb* mutant strains are reported to have impaired dissemination function, suggesting the role of HBHA in extrapulmonary dissemination of mycobacteria (Zheng et al., 2017). It is also involved in the depletion of professional macrophages by inducing apoptosis as a means of host immune invasion (Belay et al., 2016). Individuals, who are latently infected with TB exhibit a strong T-cell response to HBHA, whereas their active TB counterpart do not, emphasis that HBHA is a potential immunodiagnostic marker of latent tuberculosis and HBHA specific Th1 responses may participate in protective immunity against active TB (Hermann, & King, 2021, Parra et al., 2004, Locht et al., 2006). Memory B cells isolated from *Mtb* exposed healthcare workers were skewed towards IgA expression, indicating the role of mucosal immunity in protection (Hermann & King, 2021). Several studies in mouse model illustrated active HBHA-mediated immune-protection, where vaccine candidate with HBHA demonstrated high antigen-specific antibody serum titers and reduction in colony forming unit (CFU) in lungs and spleen of the *Mtb* infected mice. (Glatman-Freedman, 2003). HBHA is a methylated protein and the methylation pattern of HBHA facilitates the antigenicity in latently infected subjects, as well as protective immunogenicity in HBHA (Parra et al., 2004). The HBHA specific IFN-γ is informed to be generated by both of the $CD4^+$ and $CD8^+$ T cells in human and mice (Green et al., 2013). Additionally, the HBHA-specific $CD8^+$ T cells mediated bactericidal and cytotoxic activities to mycobacteria-infected macrophages which is perforin mediated (Locht et al., 2006). Considering all the evidence, HBHA could be a potential candidate for the TB vaccine (Locht et al., 2006). Although the role and nature of

these antigens in initiating mucosal immunity during natural *Mtb* infection in humans is still being undetermined, and comprehensive knowledge needs to be obtained.

3.2.5 Protective role of SIgA against TB:

Secretory IgA (SIgA) is the first line of defence against pathogens invasion at the vulnerable mucosal surfaces, initiating the systemic and mucosal immune responses that correlates with pro-inflammatory cytokines generation and pathogen eradication (Corthésy, 2007; Breedveld & Van Egmond, 2019). It is a natural antibody most abundantly produced in mucosal immunity (Corthésy, 2013). SIgA is composed by dimerization of two IgA monomers attached by disulfide bond to a J chain (Kumar Bharathkar et al., 2020). High relative concentration of IgA in secretions is explained by the presence of a single polypeptide receptor known as the polymeric Ig receptor (pIgR) accounts for the high relative concentration of IgA in secretions. During transportation of dimeric IgA across the epithelium (transcytosis), pIgR is cleaved to release secretory component (SC) and SC form disulfide bridge with dimeric IgA (De Sousa-Pereira & Woof, 2019). SC stays as part of the released IgA, which is subsequently known as SIgA at the apical surface (De Sousa-Pereira & Woof, 2019). SIgA is capable of carrying antigens, facilitates the inhibition of bacterial adherence, neutralizes toxins and viruses, and restrains the antigen uptake by epithelial cells, during transcytosis or in the mucosal fluids, and blocks the absorption of mycobacterial bacilli at the mucosal surface of the lung (Li et al., 2012).

Induction of IgA responses in the respiratory tract could have a protective role against diseases caused by respiratory infections, such as TB (Tjärnlund, 2005). SIgA against *Mtb* cell wall components may also assist in inhibiting *Mtb* binding to epithelial cells and macrophages, and hence contribute to protective immune responses against *Mtb* (Zhu et al., 2018). Various degrees of success have been achieved as sIgA has been applied in therapeutic intervention on the mucosal surface. In vitro studies demonstrated that while passing through the epithelium or by carrying pathogens can initiate protective functions, since antigen-specific dimeric IgA can neutralize endocytosed bacterial lipopolysaccharide (LPS) within epithelial cells (De Sousa-Pereira & Woof, 2019). Studies regarding immunized IgA deficient mice, have shown more susceptibility to BCG infection than immunized wild-type mice, as they illustrated a higher load in the lungs and bronchoalveolar lavage (BAL), suggesting that IgA provides the protective mechanism by impeding the entrance of bacilli into the lungs or

by regulating locally elicited pro-inflammatory immune response against mycobacterial infection in the respiratory tract (Tjärnlund, 2005).

Furthermore, early experiments utilizing monoclonal IgA and IgG against alpha-crystallin (Acr) antigen of *Mtb* in mice showed that IgA had a greater effect, as indicated by lower bacterial counts and granulomas (Tran et al., 2019). In addition, the IgA mAb has been proven to prevent infection relapse following incomplete drug treatment. In both cases, mice were given IgA mAb intranasally, and the best therapeutic results were obtained when combined with IFN-γ, a proinflammatory cytokine thought to play an important role in the suppression of *Mtb* infection (Tran et al., 2019). Another mouse model evaluated the passive protective role of IgA monoclonal antibody against *Mtb* surface antigen, where lung bacterial counts declined by 10-fold after either aerosol-or intranasal challenge. They described the protective mechanism that has been initiated through localization of IgA mAbs following intranasal provision in the lung, ADCC and stimulation of APCs, which are essential for T-cell activation (Williams et al., 2004). Furthermore, after inoculation with human IgA mAb, mice illustrated decreased *Mtb* infection rate than control mice (Breedveld & Van Egmond, 2019). Additionally, the protective role of IgA has been documented in human studies too. Belay et al (2015) demonstrated the significant higher production of anti-HBHA IgA as well as increased IFN-γ level in control than TB patient, indicated the potential protective role of IgA in naturally infected population in endemic settings. Polyclonal human secretory IgA (hsIgA) was isolated from the colostrum of healthy mothers and found to contain IgA capable of binding BCG and *Mtb* lysate. Pre-incubation or prophylactic intratracheal incubation of *Mtb* with this hsIgA decreased bacillary load and refined granuloma formation in the lungs of mice exposed to live *Mtb*. This indicates that mother and child may passively transmit antibodies that interact with *Mtb* in the mucosa and that human *Mtb* specific hsIgA can alter the course of infection (Jacobs et al., 2016). In vitro infection of human whole blood or isolated monocytes by *Mtb* was reduced in the presence of specific IgA1. Although the ephemeral protective effect, which was presumably due to the fast breakdown of the supplied IgA, this antibody was found to resist early infection in the lungs, suggesting that it could be used for immunoprophylaxis in immunocompromised people at risk of TB infection (De Sousa-Pereira & Woof, 2019).

2.3.6 Role of IgG against TB:

Most human antibodies developed against TB belong to the IgG1 and IgG3 subclasses (Jacobs et al., 2016). Mucosal IgG responses in humans are pro-inflammatory when they integrate with complement-activating and Fc-receptor I (FcRI)/FcRIII signaling functions in IgG1 and IgG3 subclasses (Chen et al., 2020). A recent study investigated the protective role of polyclonal antisera from *Mtb*-exposed healthcare personnel and demonstrated that Abs, predominantly IgG3 from latently infected or uninfected healthcare workers, had a more protective effect than TB patients against *Mtb* in an aerosol mouse model challenge (Li et al., 2017). In TB patient, IgG1 reported to be able to accelerate TNF-α production by monocytes (Pinpathomrat et al., 2021). Likewise, in a cohort study of Mexican patients, IgG against Ag85A antigen was shown to lower cavitation and had a higher probability of *Mtb* sputum clearance (Jacobs et al., 2016). Individuals who have been exposed to TB develop significant levels of IgG against *Mtb* purified protein derivatives (PPD), and healthy individuals from India had comparable antibody levels against mycobacterial surface polysaccharides compared to those with active TB disease (Jacobs et al., 2016). The possibility for the role of antibodies in people who have been in direct contact with active TB patients for a long period but are still TST-negative is that these people have higher levels of IgG against *Mtb* in their blood and that their serum can block or augment the stimulation of autologous T-cells in peripheral blood mononuclear cell (PBMC) by PPD (Jacobs et al., 2016). This suggests the potential that anti-mycobacterial antibodies aid in the control of the initial acquisition of *Mtb* infection in humans (Jacobs et al., 2016).

In vitro, it was observed that IgG antibodies targeting AM in latently infected patients increased *Mtb* absorption and intracellular death by human macrophages (Hermann & King, 2021). AM is the delipidated form of LAM, which is a primary component of the capsular layer of *Mtb*. An in vivo mouse challenge paradigm was used to further confirm this protective response, where polyclonal AM-antibodies isolated from the serum of identified subjects with latent TB infection were delivered into mice before a low concentration *Mtb* challenges. Despite the intensity of this effect being rather low, three out of four patient antibodies could minimize the bacterial burden in this investigation (Hermann & King, 2021). Additionally, surface-binding two IgG1monoclonal antibodies (16a1 and 16a6) in a murine model were able to agglutinate cultures of the pathogenic strain CDC1551 at a conc. of 100 ug/ml, in comparison to a control anti-*Mtb*, mAb was which unable to bind the surface of the similar strain (Al-Sayyed et al., 2007). This supports previous results indicating antibodies

can interact with ingested mycobacteria and restrict mycobacteria from invading the host (Jacobs et al., 2016).

3. Rationale:

An estimated 2.2 billion people globally are *Mtb*-infected (Ngo et al., 2021). However, only 5- 10% develop clinical TB, suggesting the presence of immune protection. The current knowledge of immune cells, molecules, and mechanisms of protection is still incomplete. It is believed that cell-mediated immunity, especially IFN-γ producing CD4+ T cells are critical for controlling *Mtb* infection in humans. Several decades of effort to develop an effective vaccine based on this paradigm has not been successful (Abebe, 2012). To date, TB control depends on passive case findings and DOTS. BCG has been the only available vaccine for decades, which has no effect against pulmonary TB in adults. Therefore, in order to achieve the stated goal of ending TB 2030, an effective new vaccine is required.

On the contrary, the role of Abs in protection against *Mtb* infection has been largely ignored. One of the main reasons for this misconception is the fact that *Mtb* is intracellular, and Abs cannot penetrate the cell membrane to have its effect. Nevertheless, *Mtb* has both intracellular and extracellular phases in its effective cycle, implying that Abs can be effective against the extracellular phase of the pathogen (Abebe, 2019). Therefore, identification of protective markers, especially that of Ab responses is critical for vaccine design and development against *Mtb*. *Mtb* travels through mucosal surfaces (respiratory tract) to reach the lungs. In this regard, several cell types (e.g., mucosal associated invariant T cells, natural killer cells) and molecules (sIgA, IgG) that may play a protective role against *Mtb* infection. Nevertheless, the role mucosal immune responses in general and these specific antibodies (e.g., sIgA and IgG) in protection against *Mtb* infection has not been established. In recent years, there is growing interest to develop antibody-based vaccines against TB, targeting respiratory mucosa. Mucosal vaccines against *Mtb* infection are very attractive because of safety, simplicity and public acceptance. Thus, it is utmost important to understand differences in immune parameters between clinical TB patients (susceptible) and *Mtb*-infected and/or exposed individuals with no clinical disease to design and develop a vaccine against TB.

Moreover, most of our knowledge, regarding host immune responses against *Mtb* infection comes from studies of murine models and it has been difficult to understand the true picture of host-pathogen interactions under natural conditions in human population. Hence, studies

aimed at naturally exposed and/or infected human population in an endemic setting would help to understand host factors that lead to protection against *Mtb* infection. In this regard, it is essential to assess the antibody responses to specific *Mtb* antigens to understand the true picture of host protective markers.

This study, performed in an endemic setting, measured, and compared *Mtb*-specific Abs (sIgA and IgG) in serum and saliva of pulmonary TB patients (PTB), their household contacts (HHC), and community controls (CC). Current study has generated important preliminary data on the protective role of secretory IgA and IgG against *Mtb* and role of mucosal immunity in the human population. Thus, the study expected to lay the foundation for large-scale research for vaccine design and development. Results of the study could also be used to develop an immunodiagnostic tool that can discriminate clinical TB and latent *Mtb* infection /exposure.

4. Research question and objectives:

4.1 Research questions: Two research questions have guided this study-

- Does exposure to *Mtb* induce sIgA and IgG against HBHA and LAM in mucosa and serum?
- Are anti- HBHA and anti-LAM sIgA and IgG protective against *Mtb* infection?

4.2 Objectives of the study: The main objective of the research was-

- The main objective is to assess the induction and protective role of sIgA and IgG against HBHA and LAM in *Mtb* infection.

4.2.1 Specific objectives: The specific objectives of the research were-

- To assess whether HBHA and LAM can induce the production of sIgA and IgG
- To compare the IgA and IgG level among pulmonary TB patient, their household contacts (HHCs), and community controls (CCs).
- To evaluate the potential of HBHA and LAM to induce protective mucosal immunity against *Mtb* antigen.

5. Methodology:

5.1 Study design:

The study design for this project was a descriptive cross-sectional study with a quantitative approach. Descriptive cross-sectional study is observational in nature which measures the outcome and exposure at one point in time (Setia, 2016). The study design was chosen based on the objective. The study's main goal was to measure the initiation as well as protective role of secretory IgA and salivary IgG level against HBHA and LAM antigens. In other words, we wanted to assess the induction of antibody responses (secretory IgA and IgG) against antigens (HBHA & LAM) and estimates the protective role of those antibodies among the targeted population. This cross-sectional study was selected because antibody responses against antigens were measured in a single point in time, allowing comparisons with estimations of prevalence in this population. Furthermore, cross-sectional association of antibody responses with different socio-demographic factors of interest was also evaluated. Therefore, a cross-sectional study with further quantitative analysis of the collected data was conducted.

5.2 Study setting:

The study was conducted in Addis Ababa, Ethiopia. Addis Ababa is the capital of Ethiopia, consisting of 10 sub-cities, each sub-divided into 8-11 administrative units, known as Kebeles and with a total of 99 kebeles at the time of study (Fig: 2).

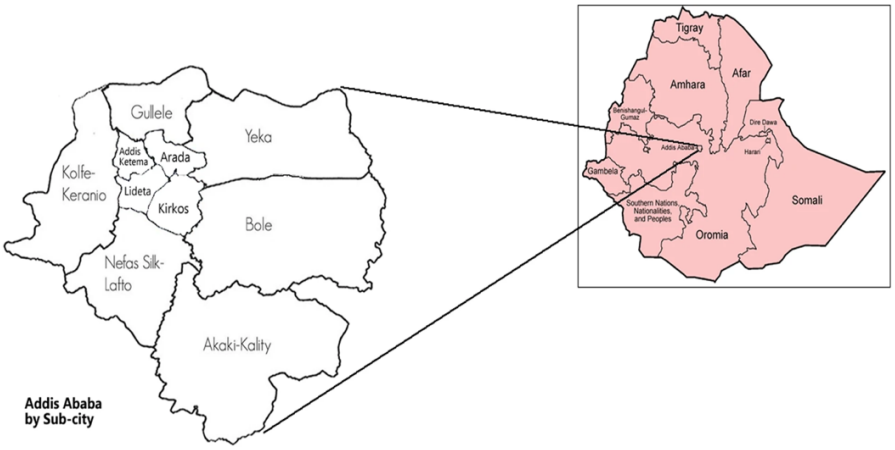


Figure 2: Map of Addis Ababa (Sinshaw et al., 2019)

The study was conducted at the Armauer Hansen Research Institute (AHRI), a biomedical research institute is based in Addis Ababa. According to the Addis Ababa Health Bureau,

close to 100 health centers exist in the city that can provide DOTS and VCT services. Of these 100 health centers, 10 were approached to participate in this study. These centers were: Woreda 01 Health centre, 09 Health Centre, Alem Bank Health Centre, Tekel-Haimanot Health Centre, Kolfe Health centres, Hana Mariam Health Centre, Meshualekia Health Centre, Addis Raey Health Centre, Kality Health Centre, and Lomi Meda Health Centre. These sites were selected based on TB burden and proximity to AHRI laboratory. In TB clinics, TB patients are directly observed taking their daily treatment doses and at voluntary HIV counseling and testing (VCT) clinics in selected health centers in Addis Ababa, Ethiopia. The health centers register and keep records of TB patients and provides both diagnostic services and DOTS for TB patients. Anti-TB drugs are provided free of charge by nurses for 6 months, and the nurses supervise the patients three times in a week during intensive phase and once in a week during continuation phase until patient complete their treatments under routine DOTS program.

The DOTS and VCT clinics were selected to target and recruit TB patients, their household contacts and apparently healthy individuals. Briefly, 30 smear positive pulmonary TB patients (PTB), age between 18 and 50, were identified and recruited into the study. Additionally, 30 adults living in the same house with the pulmonary TB patient were invited to participate in the study as household contacts (HHC). Apparently healthy individuals (30, CC) were randomly selected and recruited from VCT centres from the selected health facilities.

5.3 Study population & data collection:

5.3.1 Sample size:

A total of 90 participants were divided into three study groups, 30 participants in each group. The sample size from each study group was selected based on a convenient sampling approach and according to the central limit theorem, 30 is considered as a minimum sample size that allows statistical analysis and is also appropriate for validity to observe an anticipated difference between the study groups (Islam, 2018). All participants were adults, aged between 18 and 50 years. Individuals with immunosuppressive conditions (pregnancy, diabetes etc.) or HHC with previous history of TB disease or CC with history of TB contacts or a previously known history of co-infections were excluded from the study by questionnaire. Participants were enrolled into the study based on the eligibility criteria (Table 1). Screening for HIV was done according to the Ethiopian national guidelines, where TB

patients were offered the test through provider-initiated counselling and testing (PICT) platforms in TB clinics and for household contacts and controls through VCT platforms.

5.3.2 Selection of study participants:

A total of 99 participants were enrolled into the study, of which 30 were active pulmonary TB patients (PTB), 33 were household contacts (HHC), and 36 were community controls (CC). Among them 9 participants, 3 HHC and 6 CC were excluded due to exceeding age limit, previous TB history, and missing QuantiEFRON (QFT) samples.

In Ethiopia, including Addis Ababa and its surrounding area, TB suspects are referred to DOTS centers for examination and treatment. At DOTS centers, suspects undergo clinical and x-ray examinations, followed by sputum smear microscopy or Gene- Xpert test. Gene-Xpert or sputum smear positive suspects (n =30) were recruited into the study with written consent (Appendix I). Patients were considered TB-positive when at least two of three consecutive sputum smear examinations were positive for acid fast bacilli. Gene-Xpert was used as a rapid diagnostic test for TB detection and rifampicin resistance testing in direct smear negative cases. Individuals receiving standards anti-TB treatment for two weeks were included to minimize altered immunity and effects of anti-TB treatment on antibody response. Routine physical and clinical examinations were done.

HHC were those family members who shared the same household with TB index cases. In order to have effective contacts, the inclusion criterion was individuals who lived with TB patients (n =30) for three or more months and had given written consent (Appendix II). None of the contacts had clinical symptoms or findings indicative of active TB. Any HHC with previous history of TB were excluded.

CC were recruited randomly among persons attending general physical examination at the VCT centers of the selected health facilities. Healthy individuals, with no clinical symptoms or evidence of active TB (ATB) and no prior exposure to TB or any immunosuppressive condition (pregnancy, diabetes, HIV) or other co-infections were included as community controls. These controls did not share a household with TB index cases or did not engage in random social gathering (having coffee together) with known TB patients or their HHC. Physical examination was carried out initially. All female participants were screened for pregnancy before including into the study. Additionally, all participants were screened for

HIV before inclusion, the BCG vaccination status was confirmed through visual inspection of BCG scar in the HHC and CC arms, and only HIV negative participants were enrolled.

5.3.3 Data collection:

Participants' clinical, physical, and socio-demographic data were collected using close-ended structured questionnaires (through interviews). Data were collected in English; however, the questionnaires were translated into Amharic for participants and conveyed to participants by study nurses at the respective health centers.

Study nurses were given instructions regarding eligibility criteria (as seen in Table 1) to enroll participants, as well as on collection of clinical samples and questionnaire data. Moreover, the participants were informed about the objectives, study procedures, and written informed consent was obtained. The content of the questionnaire, aim and purpose of sample collection, procedures of sample collection and confidentiality perspectives were also explained to the interviewee. The interviewees were informed that he/she/they can withdraw from the study any time if they want and all information, including biological samples would be deleted or destroyed.

Table 1:Eligibility criteria for the study population (February - July 2022)

Inclusion criteria	**Exclusion criteria**
TB patients	
• Newly identified Pulmonary TB (PTB) patients.	• Extra-pulmonary TB (PTB) patient
• Bacteriologically confirmed PTB cases (either by AFB or Gene-Xpert)	• Smear negative TB patient
• DOTS initiated within 2 weeks and no clinical sign or symptoms of immunosuppressive condition (HIV, diabetes, or autoimmune disease).	• MDR, relapse, defaulters, retreatment cases and cases with prior history of TB or any immunosuppressive condition (HIV, diabetes, or autoimmune disease).

Household contacts	
• Contacts of bacteriologically confirmed PTB patients.	• Contacts with prior history of TB (active TB).
• Living with index TB patients for at least past 3 months prior initiation of treatment of the TB index case.	• Not Living with smear-positive TB case.
• No clinical sign or symptoms of immunosuppressive condition (HIV, diabetes, or autoimmune disease).	• Presence of clinical sign or symptoms of co-infection or immunosuppressive condition (HIV, diabetes, or autoimmune disease).
Community controls	
• Living in the same community.	• Previous history of TB.
• Apparently healthy individuals without no known prior or current history of TB illness.	• Contacts with pulmonary TB patients.
• No clinical sign or symptoms of immunosuppressive condition (HIV, diabetes, or autoimmune disease).	• Presence of clinical sign or symptoms of co-infection or immunosuppressive condition (HIV, diabetes, or autoimmune disease).

5.3.4 Sample Collection and preparation and lab analyses:

Blood and saliva samples were collected up to 2 weeks of initiation of anti-TB treatment for PTB patients. Strict aseptic sample collection procedures and waste disposal were conducted by experienced nurses and any used/unwanted biological material, syringes, tubes, and needles were disposed into designated septic tanks at the health centers as well as AHRI.

Blood sample:

Blood sample was drawn from participants in the three respective groups to determine antigen-specific antibody levels by serum IgA and IgG ELISA assay. Primarily, 4ml of whole blood was collected in serum separating tube (SST) with clot activator and gel. Sample was left undisturbed at room temperature for 20-30 minutes to complete clot formation. The clot was removed by centrifuging for 10 minutes at 3,000 rpm at (18-20°C). Using clean pipette technique, ≃2ml of serum was aliquoted into 4 labeled small eppendorf tubes (500µl in each tube) and stored at -80°C until use in the future.

In addition, about 6ml of whole blood was collected in a single generic blood collection tube that contains lithium heparin as an anticoagulant and transported to AHRI's laboratory at ambient temperature, within 2-6 hours of blood collection. Upon arrival, approximately 1ml of whole blood was transferred to 4 individual 1ml QuantiFERON tubes as supplied by the manufacturer (Qiagen GmbH, Germany), and mixing was performed by shaking the tubes 10 times just firmly enough to ensure that the entire inner surface of the tube is coated with blood. Later, the tubes were incubated at 37°C for 16 to 24 hours. The tubes were- a nil control tube (negative control), TB antigen tube 1(TB1) (containing peptides from ESAT-6 and CFP-10), TB antigen tube 2(TB2) (containing peptides from ESAT-6 and CFP-10 as well as additional peptides to stimulate CD8+ T cells), and a mitogen (PHA) control tube. Following incubation, the tubes were centrifuged at 2,000rpm for 15 minutes at 20°C, the plasma was removed. Finally, supernatants were harvested and stored at -80°C until further testing for quantification of interferon-γ using enzyme-linked immunosorbent essay (ELISA).

Saliva collection and dilution:

Resting drool technique was used to collect the whole mouth saliva from the participants. Participants were instructed to clean their mouth with clear water and were informed to suspend eating one hour before collecting the sample to eliminate food debris and unwanted contamination which could hamper the analytical the lab analysis. At the beginning of sample collection, participants were asked to sit upright position and tilts their head slightly downwards to naturally flow the saliva down in the tube. Approximately 2-3ml of saliva was collected in falcon tubes. Later, the unprocessed sample was transferred into two microcentrifuge tubes (1ml in each tube) for the centrifugation. All saliva samples were centrifuged at 10,000 ×g for 10 minutes at 4°C. The supernatant was then transferred into two

newly labeled eppendorf tubes and stored at -80°C until further assayed. Processed saliva samples were diluted according to the to the manufacturer`s instructions (IBL International, Hamburg, Germany). Each sample was prepared by diluting the supernatant fluid 1:20 with diluted assay buffer (ie: 50 µl up to 1 ml). Then every solution was mixed gently by leaving it at least for 5 minutes on a rotating shaker and diluted further 1:50 with diluted assay buffer (ie: 20 µl up to 1 ml), using manufacturer's instructions. (IBL International, Hamburg, Germany). Final dilution obtained 1:1000.

5.3.5 QFT-GIT ELISA:

The QuantiFERON-TB GOLD PLUS test was used to determine *Mtb* infection in HHC and CC. Blood sample collection (whole blood), incubation and subsequent ELISA assay was done according to the manufacture's instruction (Qiagen GmbH, Germany). Briefly, plasma samples were removed from -20°C freezer and thawed at room temperature and were assayed for quantitation of IFN-γ release using ELISA. The optical density of each well was measured and analyzed using the QFT PLUS analysis software (Qiagen GmbH, Germany). A positive test result was determined at antigen −nil ≥ 0.35 IU/mL and ≥ 25% of the nil sample, whereas a negative test was defined as antigen–nil < 0.35 IU/mL or < 25% of nil and mitogen ≥ 0.5 IU/mL.

5.3.6 Antigens:

Selected *Mtb* antigens (LAM and HBHA) were used to measure and compare antigen-specific antibody response in the three groups of participants. We selected these antigens, as they are few of the most characterized *Mtb* antigens to elicit strong immune response in TB.

Purified LAM (NR- 14848) from *Mtb*, strain H37Rv, was obtained from Biodefense and Emerging Infectious Research Resources Repository, National Institute of Allergy and Infectious Diseases, National Institute of Health, Manassas, USA. Similarly, purified HBHA was received from Professor Tom Ottenhoff and Professor Kees L Franken's laboratory, Department of Infectious Diseases, Leiden University Medical Center, Leiden, The Netherlands.

5.3.7 Antibody ELISA:

Serum IgA and IgG as well as salivary IgA were measured against LAM and HBHA using ELISA, following a series of optimization to determine the optimal concentration. Briefly,

LAM and HBHA-specific IgA and IgG levels were measured using sandwich ELISA procedure. High binding costar 96-well plates (Corning, USA) were coated with HBHA, and LAM (10µg/ml) which were diluted in coating buffer (100µl/well) and incubated overnight at 4°C. Plates were emptied and blocked with PBS with 0.05% Tween 20 and 0.1% BSA (Mabtech, Nacka Strand, Sweden) and incubated 1 hour at room temperature (RT). Then, plates were washed 5 times with PBS containing 0.05% Tween 20 (300µl/well). After washing, 100µl of samples diluted 1:10 in incubation buffer (PBS with 0.05% Tween 20 and 0.1% BSA) were added and plate was incubated at RT for 2 hours.

Simultaneously, another plate was coated with capture mAb MT57 for IgA and MT145 for IgG (Mabtech, Nacka Strand, Sweden) diluted to 2 µg/ml in PBS (pH 7.4) and incubated overnight at 4°C. Plates were then emptied and blocked with PBS with 0.05% Tween 20 and 0.1% BSA. After washing with PBS containing 0.05% Tween 20, 100µl of diluted serum samples were added with similar dilution concentration of 1:10 for both IgA and IgG, 100µl of standards and previous samples stimulated at the antigen plate were also transferred to antibody coated plate. The plates were washed and 100µl of detection mAb MT20-ALP (IgA) and MT78-ALP(IgG) (Mabtech, Nacka Strand, Sweden) diluted in 1:1000 in PBS with 0.05% Tween 20 and 0.1% BSA) were added into each well of respective plates. Plates were incubated for 1 hour at RT and after washing, 100µl/well of pNPP substrate tablets diluted in PBS with 0.05% Tween 20 and 0.1% BSA was added and incubated for approximately 60 minutes. Optical density was measured in ELISA plate reader (SoftMax® Pro 7 Software version 7.0.3 for Windows) at 405nm and a reader capable of subtracting a reference wavelength of between 570 and 650nm. Optical density (OD) was adjusted by subtracting the mean reference OD well from the negative control well. Finally, both antigen-stimulated and un-stimulated antibody OD values were included in the analysis.

For salivary IgA, precoated anti-human monoclonal IgA antibody microtiter plates were used as per the manufacture's instruction (IBL International, Hamburg, Germany). Diluted saliva samples (1:1000) according to the manufacture's instruction (IBL International, Hamburg, Germany) were stimulated with LAM and HBHA (10µg/ml) in separate antigen coated plates following the similar preparation of serum IgA and IgG. Then, 25µl of diluted saliva samples (1:1000) as well as 25µl of stimulated saliva samples from the antigen plates were transferred to the precoated plates along with 25µl standards and 100µl diluted conjugates (horseradish peroxidase conjugated polyclonal anti-human IgA antibody) and incubated at RT for 1 hour.

Then, plates were washed three times with 300µl diluted (1:50) wash solution. 100µl of TMB substrate were added in the plates and incubated for 15 minutes in the dark at room temperature. Reaction was stopped using 100µl of stop solution (sulfuric acid). Optimal OD value was measured in ELISA plate reader (SoftMax® Pro 7 Software version 7.0.3 for Windows) at 450nm against a reference wavelength of 620-630nm within 5minutes. Mean reference OD well value was subtracted from the negative control well. Finally, both antigen-stimulated and un-stimulated antibody OD values were included in the analysis.

5.3.8 Data Analysis:

All antibody data were presented as OD values in this study and were considered as dependent variables, whereas variables including clinical presentation and socio-demographic data (such as age, gender occupation, presence /absence of BCG scar, HIV status, COVID-19, and QFT-GFT test) were considered as independent variables. The socio-demographic characteristics were descriptively summarized among study groups using frequencies and percentages. Associations between categorical variables were assessed using the Chi-square test, if expected counts were ≥ 5, otherwise the Fisher's exact test was used. Numerical data were summarized using the median and interquartile range (IQR) after checking for normality using the Shapiro Wilk test. For antibody data analysis, values from negative control wells were subtracted from antigen-stimulated and unstimulated OD values to adjust for non-specific background. Data were not normally distributed, and non-parametric statistics were used to compare groups. Kruskal-Wallis test (One-Way ANOVA) with Dunn's multiple comparisons test were used to compare antibody responses among groups. Multiple regression analysis was performed to assess the association between antibody levels and socio-demographic variables. P-values less than 0.05 were considered statistically significantly. Analyses were performed using IBM SPSS Statistics (version 28) predictive analytics software and GraphPad Prism version 8.0.0 for windows (GraphPad Software, San Diego, California USA, www.graphpad.com).

5.3.9 Ethics statement:

The study was approved by the Addis Ababa Health Bureau and AHRI/ALERT Ethics Review Committee from Ethiopia, Regional Committees for Medical and Health Research Ethics, South-East Norway (REK- Sør-Øst) and Norwegian Centre for Research Data (NSD) in Norway.

Identity of the participants was kept anonymous and other people enlisted to the study were trained to maintain this anonymity. All participants were informed about the study and gave written informed consent before enrolled into the study. The content of the questionnaire, aim and procedures of sample collection, and confidentiality and withdrawal conditions were explained to the participants. All the information including data set and questionnaire were protected in password coded database. For the data storage, we used TSD service for sensitive data, which is a platform in University of Oslo network for collecting, storing, analyzing and sharing sensitive data. It ensures high security for the data storage and management. It can be assessed through any part of the world with stable internet connection. Through TSD, we directly transfer and upload this research data, field notes, sample analysis from the fieldwork, all ensuring data protection, backup and high computing service. Only the principal investigator has access to all confidential data storage. Non-anonymized data that matches participant names to coded number were to be only accessible to the researchers.

6. Results:

Sociodemographic variables:

From February to July 2022, through inclusion and exclusion criteria, 90 participants were consecutively included into the study, of which 30 were PTB patients, 30 were HHC to TB patients, and 30 were CC. A comparison among the three study groups by socio-economic factors is outlined in Table 2. All three study groups had a higher proportion of female participants (53.3%, 80%, and 66.7%, respectively). No significant statistical differences were observed among the three study groups in age, gender, and prior Covid-19 test results. In addition, TB patients had the lowest percentage of BCG scar (26.7%), whereas the control groups had the highest percentage of the BCG vaccination history (60%). The differences were statistically significant (P= 0.020), and the groups also vary in terms of occupation (P=0.030). None of the participants in the three groups were tested HIV positive, and more than half of the contacts (60%) had never undergone a HIV test or were unaware of their own HIV status. Furthermore, in comparison to individuals who had prior history of interaction with TB patients (HHC) other than the index cases included in the study (30%), those who reported to have no history of exposure (CC) had a higher percentage of QFT-GFT positivity (46.7%). However, the difference was not significant statistically (P = 0.184). Furthermore, no apparent association was found between antibodies level and sociodemographic variables

(data not shown), except for serum IgA, which levels poorly correlated with prior Covid-19 status, ($R^2 = 0.26$, P = 0.006).

Table 2:Socio-demographic variables of study groups

Variables	PTB patients	Household Contacts	Community Controls	P-value
Age in years (Median and IQR)	26 (23.8-32.8)	34(24.8-39.3)	30(25-40)	0.074
Gender				
Male	14 (46.7%)	6(20%)	10(33.3%)	0.091
Female	16(53.3%)	24(80%)	20(66.7%)	
Occupation				
Civil servant (Gov't)	2(6.7%)	9(30%)	8(26.7%)	0.030
Private worker	15(50%)	7(23.3%)	13(43.3%)	
Student	0(0%)	1(3.3%)	0(0%)	
Unemployed	9(30%)	8(26.7%)	9(30%)	
Others	4(13.3%)	5(16.7%)	0(0%)	
BCG Scar				
Present	8(26.7%)	15(50%)	18(60%)	0.020
Absent	22(73.3%)	13(43.3%)	12(40%)	
Indeterminate	0(0%)	2(6.7%)	0(0%)	
HIV test				
Positive	0(0%)	0(0%)	0(0%)	-
Negative	30(100%)	14(46.7%)	30(100%)	
Not tested/don't know	0(0%)	16(53.3%)	0(0%)	
Prior COVID-19 test result				
Yes	3(10%)	1(3.3%)	4(13.3%)	0.383
No	27(90%)	29(96.7%)	26(86.7%)	
QFT-GFT test result				
Positive	N/A	9(30%)	14(46.7%)	0.184
Negative		21(70%)	16(53.3%)	

* N/A= Not available for the TB patients

*IQR= Interquartile Range

IgA responses to HBHA and LAM among pulmonary TB patients, household contacts and community controls in saliva

To compare the antibody responses against HBHA and LAM among three groups, we used non-parametric test- Kruskal-Wallis test with Dunn's multiple comparison test. Unstimulated and LAM- and HBHA-stimulated IgA levels were measured in both saliva and serum samples, where IgG was only measured in serum. Initially, we compared the HBHA and LAM- stimulated antibody responses among the groups (Fig: 3- a,b,d,e) and afterwards, we compared Ag-stimulated antibody responses with Ag-unstimulated antibody responses (Fig: 3-e,f). When HBHA and LAM- stimulated IgA were compared among the three clinically categorized groups, HHC had higher IgA responses against HBHA compared to PTB patients ($P < 0.05$). Following this, HHC and CC were stratified based on their QFT test results and new subgroups, namely, QFT-positive and negative were made irrespective of the clinical categorization. Overall, QFT-negative, and positive groups had comparable anti-HBHA antibody responses in saliva. The median saliva level of HBHA-specific IgA was higher in both QFT- positive and negative groups compared to PTB patients. However, the differences were not statically significant ($P = 0.79$) (Fig.1- b). On the other hand, no significant differences were noted in the saliva of PTB patients, HHC, and CC for LAM-specific IgA (Fig: 3-d). No significant differences were also observed when groups were compared based on their QFT responses and when compared with their Ag-unstimulated counterparts (Fig:3-e,f).

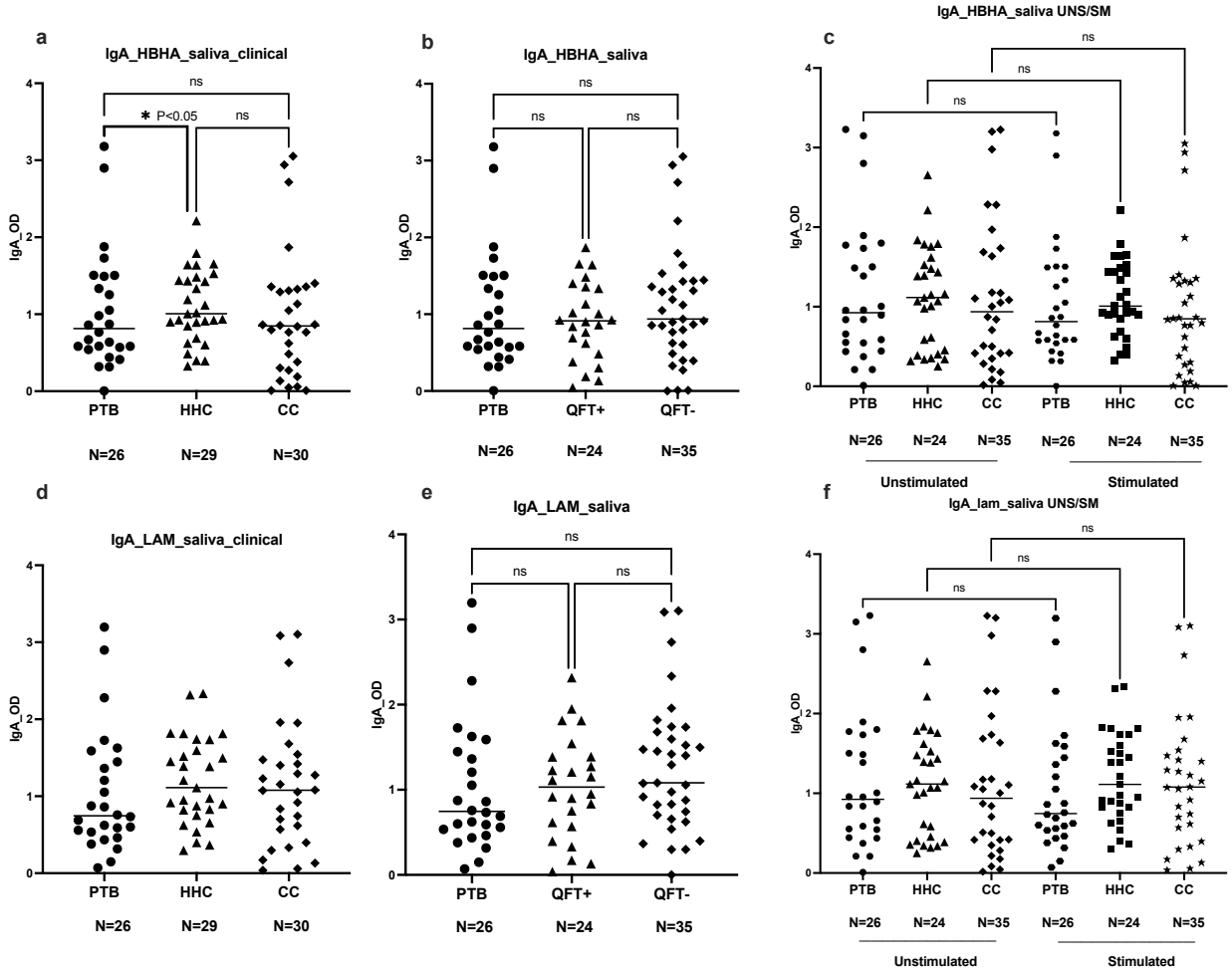


Figure 3:Scatter plots showing comparisons of the IgA responses to HBHA and LAM in saliva.

Scatter plots having comparison of the antibody response to HBHA (a) and LAM (d) among the three clinical groups, comparison of the differences among active TB patients, QFT-GFT positive and negative groups (b,e), as well as comparison between Ag-unstimulated and stimulated IgA responses among the groups (c, f). Results are individual responses and antibody responses are expressed as OD value. Error bars are medians. Abbreviation: PTB, pulmonary TB patients; HHC, household contacts; CC, community controls; QFT +ve, latently infected individuals; QFT-ve, Mtb uninfected individuals. *p <0.05, **p <0.01.

IgA responses in serum to HBHA and LAM among pulmonary TB patients, their household contacts and community controls

In the case of HBHA-stimulated IgA responses in serum, there were significant differences among the three clinically categorized groups. HHC had higher IgA responses to HBHA than

PTB patients. QFT-GFT negative groups, in addition, had significantly higher anti-HBHA antibody responses compared to PTB patients (P <0.05) (Fig:4-b). In addition, HBHA-stimulated IgA responses were significantly higher in HHC compared to HBHA- unstimulated IgA responses in HHC and CC.

Similarly, IgA responses to LAM in serum were compared among the study groups (Fig: 4). In comparison to PTB patients and their negative equivalents, QFT-GFT positive group showed higher median serum level of anti-LAM antibody responses, however, the differences were not statistically significant (Fig:4- e). Furthermore, there were no apparent differences among the three clinically classified groups and when compared with LAM-unstimulated IgA groups (Fig:4-d, f).

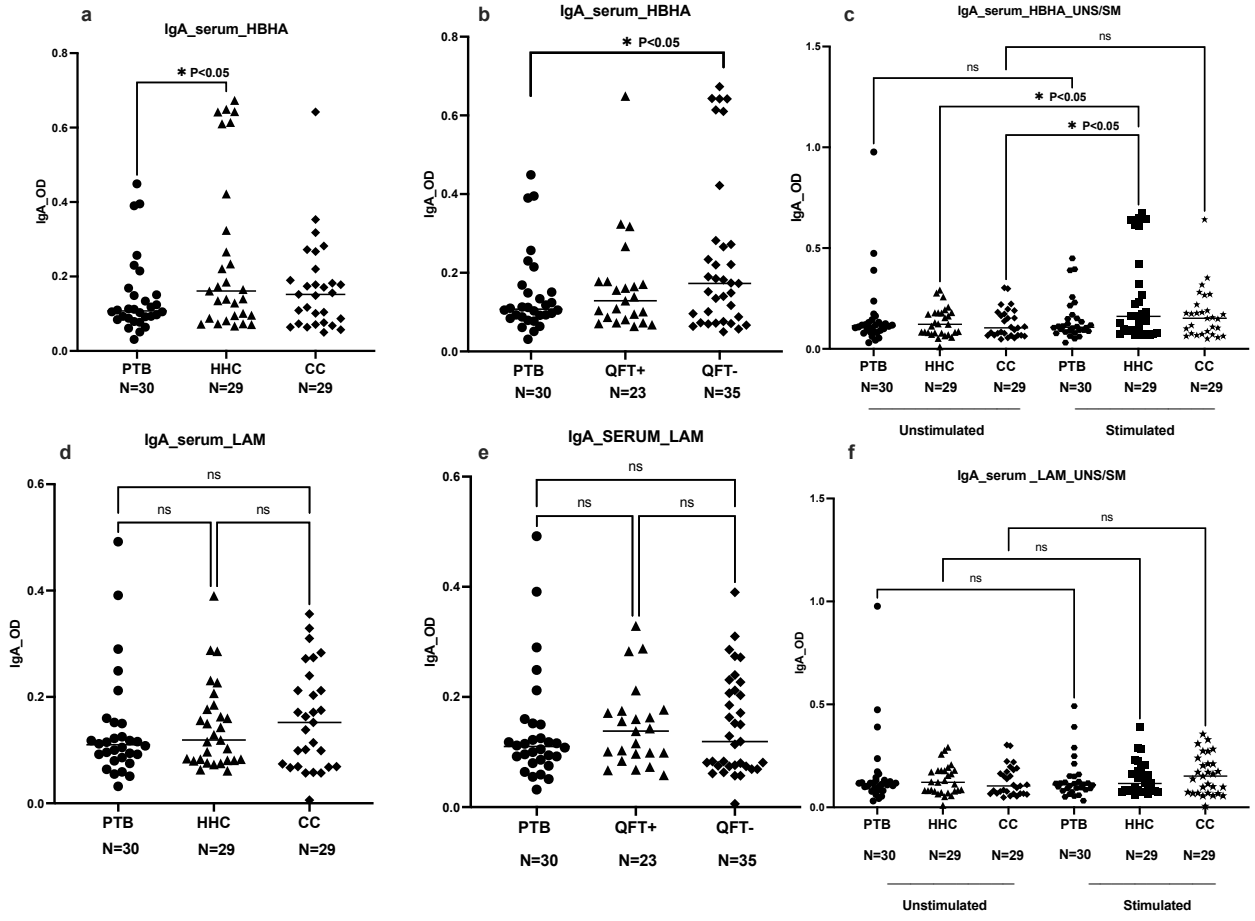


Figure 4: Scatter plots showing comparisons of the IgA responses to HBHA and LAM in serum.

Scatter plots having comparison of the antibody response to HBHA (a) and LAM (d) among the three clinical groups, comparison of the differences among active TB patients, QFT-GFT

positive and negative groups (b,e), as well as comparison between Ag-unstimulated and stimulated IgA responses among the groups (c, f). Results are individual responses and antibody responses are expressed as OD value. Error bars are medians. Abbreviation: PTB, pulmonary TB patients; HHC, household contacts; CC, community controls; QFT +ve, latently infected individuals; QFT-ve, *Mtb* uninfected individuals. *p <0.05, **p <0.01.

IgG responses in serum to HBHA and LAM among pulmonary TB patients, their household contacts, and community controls in serum

For IgG against HBHA, the clinical groups showed significant differences in antibody response in serum. Both HHC and CC had significantly higher HBHA-specific IgG responses compared to PTB patients (P <0.05, P <0.01, respectively) (Fig: 5-a). Furthermore, QFT-positive and negative groups also showed significantly higher IgG responses against HBHA compared to patients with pulmonary TB (P<0.05, P<0.01) (Fig: 5-b). No apparent differences were observed in IgG responses between HBHA-stimulated and HBHA-unstimulated groups.

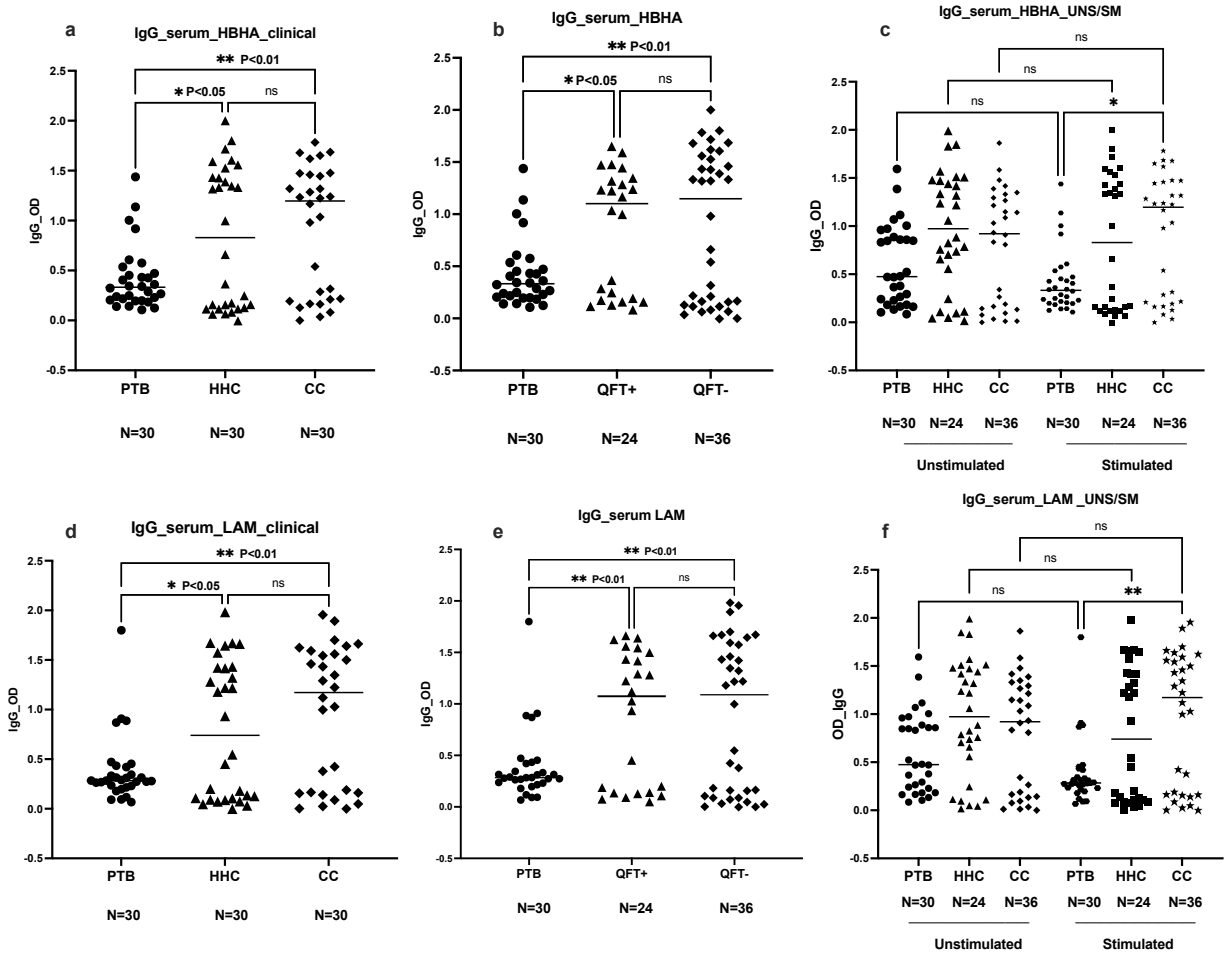


Figure 5:Scatter plots showing comparisons of the IgG responses to HBHA and LAM in serum.

Scatter plots showing comparison of the antibody response to HBHA (a) and LAM (d) among the three clinical groups, comparison of the differences among active TB patients, QFT-GFT positive and negative groups (b,e), as well as comparison between Ag-unstimulated and stimulated IgG responses among the groups (c, f). Results are individual responses and antibody responses are expressed as OD value. Error bars are medians. Abbreviation: PTB, pulmonary TB patients; HHC, household contacts; CC, community controls; QFT +ve, latently infected individuals; QFT-ve, *Mtb* uninfected individuals. *p <0.05, **p <0.01, ***p <0.001, ****p <0.0001.

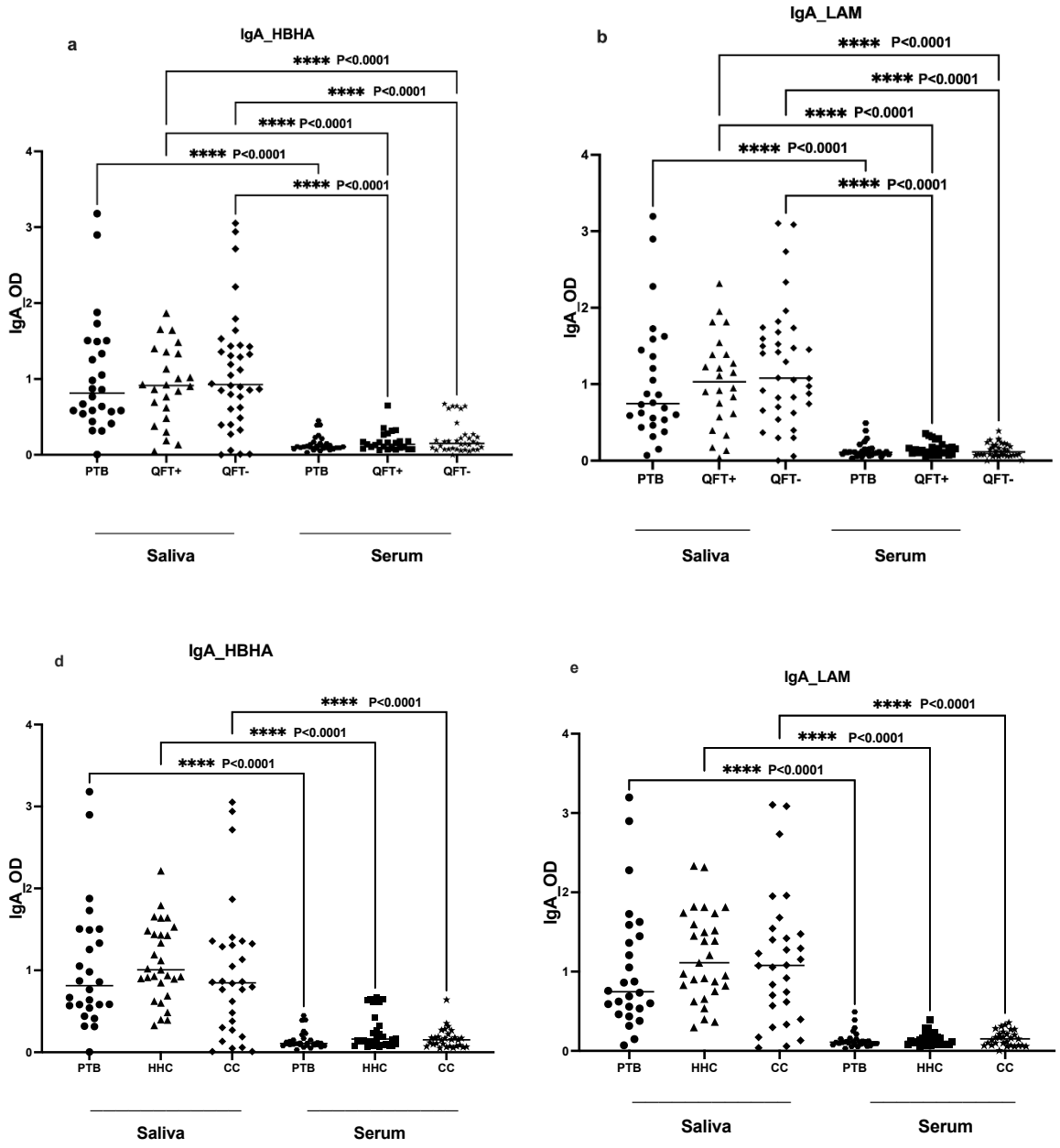
IgG response to LAM in serum is summarized in Fig. 5. Among clinically categorized groups, in comparison to HHC and CC, PTB patients had a significantly depressed IgG responses to LAM ($P < 0.05$, $P<0.01$) (Fig. 3-d). Additionally, in contrast to PTB patients and QFT-GFT positive and negative groups demonstrated statistically stronger IgG against LAM in serum ($P<0.01$) (Fig.3-e). Furthermore, LAM-unstimulated IgG responses did not vary significantly in comparison to LAM-stimulated IgG responses among groups.

Group comparison of IgA response to HBHA and LAM in saliva and serum

In saliva, the anti-HBHA IgA response of PTB patients had significantly higher responses than in serum ($P < 0.0001$) (Fig. 4-a, d). Both QFT- positive and negative groups had higher IgA responses to HBHA in saliva than their IgA responses in serum ($P <0.0001$, $P <0.0001$, respectively) (Fig. 4-b). Additionally, differences between anti-HBHA IgA responses of HHC and CC in serum and saliva also were statistically significant ($P <0.0001$).

Regarding IgA response to LAM, a similar pattern was shown. PTB patients had significantly higher anti-LAM IgA responses in saliva than in serum ($P <0.0001$) (Fig. 4-b). In comparison to QFT- positive and QFT-negative IgA responses in serum, both QFT- positive and QFT-negative showed higher IgA response to LAM in saliva ($P <0.0001$, $P <0.0001$). Moreover, HHCs and CCs responded to IgA considerably higher in saliva than they did in serum ($P <0.0001$, $P <0.0001$). In addition, heat mapping of antibody OD values with discontinuous color shading for clinical and QFT categorization, ranging from yellow to green to dark blue.

The heatmaps highlighted that majority of the HHC and CC as well as QFT-positive and QFT-negative groups had higher IgA and IgG levels in serum and saliva. PTB patients had lower levels of antibody responses which are indicated by the darker blue shades, except for very few patients both in clinical and QFT categorization, who had yellow and green shades, representing higher antibody responses.



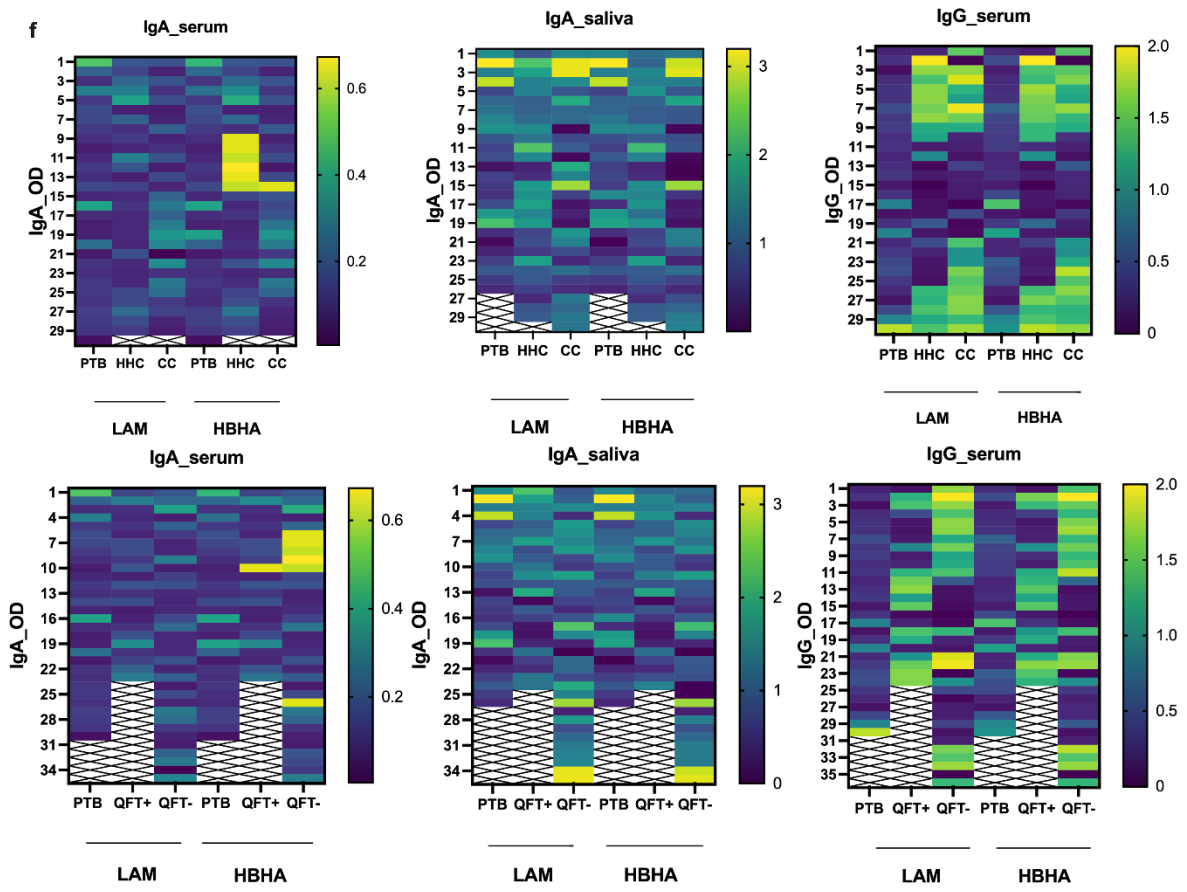


Figure 6: Scatter plots and heat maps showing comparison of the IgA responses to HBHA and LAM in saliva and serum among group.

Scatter plots having comparison of the IgA response to HBHA and LAM among QFT positive and negative groups (a, b), among three clinically categorized groups (c,d) in saliva and serum, and (f) heat maps summarizing the antibody (IgA & IgG) OD values in both saliva and serum among three clinical categorized groups (study groups in column and antibody OD values in row). Results are individual responses and antibody responses are expressed as OD value. Error bars are medians. Abbreviation: PTB, pulmonary TB patients; HHC, household contacts; and CC, community controls; QFT +ve, latently infected individuals; QFT-ve, *Mtb* uninfected individuals. *p <0.05, **p <0.01, ***p <0.001.

7. Discussion:

The present study investigated IgA and IgG responses to *Mtb* antigens, LAM and HBHA, in saliva and serum and compared antibody responses among PTB patients, their household contacts, and community controls in a TB endemic setting. In this study, we observed a significantly higher anti-HBHA IgA response in HHC than PTB patients in saliva. Thus, for the first time, our study showed the protective role of secretory IgA against HBHA of *Mtb* in saliva. Previously, Belay et al. (2016) had shown the relatively higher anti-HBHA IgA responses in contacts in serum, highlighting the protective role the HBHA-induced IgA against TB. Furthermore, the results showed that in contrast to PTB patients, QFT- positive and QFT-negative groups had the higher median IgA and IgG responses against HBHA and LAM. Additionally, HHC and CC had significantly higher levels of IgA and IgG responses against HBHA and LAM in both saliva and serum compared to PTB patients, who had the lowest level, suggesting the protective role of IgA and IgG against *Mtb* infection. Li et al. (2017) studied the protective function of polyclonal antisera from *Mtb*-exposed healthcare workers (HCWs), where antibodies, mainly IgG3, from latently infected or uninfected HCWs demonstrated a protective role in a mouse model of aerosol infection. In addition to the mouse model, Li et al. (2017) also studied antibodies in an in vitro human whole blood assay and, in both experiments, antibodies from a group of donors with LTBI and HCWs (exposed and uninfected) showed restricted *Mtb* growth in comparison to patients with active TB (Li & Javid, 2018). Another recent study compared the differences in antibodies between active TB patients and individuals with latent *Mtb* infection, in which IgG from LTBI showed superior functional properties and suppressed *Mtb* growth more effectively in macrophages than IgG from those donors with active TB (Lu et al., 2016). Zimmermann et al. (2016) reported of separating plasmablasts from pulmonary TB patients and *Mtb*- exposed HCWs and identified antibodies (IgA and IgG) specific for the surface antigens LAM and HBHA. *Mtb*-specific plasmablasts and memory antibodies derived from HCWs showed higher affinity specific for surface antigens, LAM and HBHA. In addition, a significant Ab response specific to TB69 epitope of the 14-KDa antigen was observed in healthy nurses working in a TB hospital (Rijnink et al., 2021). These findings are consistent with other studies that found antibodies from *Mtb*-exposed contacts and controls to be more protective than those from active TB patients (Tran et al., 2019). Furthermore, a study investigating Rv2659c and Rv3128c-specific IgA and IgG memory B cell responses between LTBI individuals and an active TB group demonstrated that IgA MBCs specific to antigen Rv2659c were substantially higher in LTBI

individuals (Soe et al., 2021). Together, the findings from these studies strongly indicate the protective role that antibodies play in preventing the development of active or latent TB in humans.

Majority of population who are infected with *Mtb* may not become active TB patient during their lifetimes, hence; they may be regarded as having acquired immunity (Temmerman et al., 2005). The evidence that 9 out of 10 humans tend to control *Mtb* infection in a stage of clinical latency supports the presence of natural immunity against TB (Jacobs et al., 2016). During the enrollment period of our study, participants in the CC group had no symptoms or history of clinical infection or as far as known, had never been in contact with TB patients. However, 46.7% of the community controls showed latent infection based on the QFT test results (Table 2). Owing to living in a TB endemic region, constant social interactions, and a lack of protective measures while interacting with TB patients, it is relatively harder to control for exposure to *Mtb* and these could be potential confounding factors.

Alternatively, household contacts who were reported to be exposed to active TB patients showed diverse immune response. Many of HHCs remained healthy despite being exposed, those who were infected may have effectively controlled infections, while others may not. Thus, these groups provide immunological evidence of exposure to *Mtb,* but are still able to resist *Mtb* infection, suggesting the presence of possible natural resistance to *Mtb* infection. Likewise, a significantly higher antibody response was found against a non-immunodominant epitope of Acr antigen in a previous study of nurses exposed to TB (Jacobs et al., 2016). One possible explanation could be that despite considerable exposure to the bacillus, these people generate antibodies against *Mtb* that resolve infections (Jacobs et al., 2016). Tubercle bacilli are known to discharge in higher quantities from necrotic lesions, and antibodies may come into contact with the extracellular bacilli and modify their FcR-mediated ingestion by non-infected macrophages or dissemination pattern and regulate macrophage activation (Tran et al., 2019). Antibodies with improved Fc effector pattern have been demonstrated in latent TB infection, which further stimulates macrophages to eliminate intracellular bacteria (Abebe, 2019). Similarly, utilizing sera from LTBI and health care worker participants, neutralization of the bacilli was shown in an in vitro study (Melkie et al., 2022). In a recent infant case control study, greater IgG anti-Ag85A titers were linked to a lower chance of progressing to active disease, which implies that initial *Mtb* infection in humans could be contained by anti-mycobacterial antibodies (Fletcher et al., 2016; Jacobs et al., 2016). Additionally, antibodies that attach to cell-surface antigens are able to induce opsonization, which affects the ability of

phagocytic cells to uptake bacteria and intracellular transportation (Kumar et al., 2015). This notion has been further augmented by a study in India that documented that opsonizing antibodies isolated from healthy subjects were able to prevent the *Mtb* h37Rv growth in macrophages and improved intracellular killing by elevating LAMP-1 and iNOS trafficking to the phagosome along with phagolysosome acidification (Kumar et al., 2015). An alternative explanation could also be that existing assays for LTBI simply failed to pinpoint the immunological exposure mediators in those subjects (Li & Javid, 2018).

Moreover, none of the patients with active pulmonary infection in our study had shown protective antibody responses. Although it is challenging to offer a conclusive answer, one possibility is that being virulent factors, LAM and HBHA might inhibit the generation of antibodies during active infection (Belay et al., 2016). On the contrary, majority of the HHC and CC generated higher antibody responses, except for those, who had lower IgG responses to LAM. These groups of people may have incipient TB infection, who have chances to develop TB disease in near future without any prevention (Drain et al., 208), and follow up studies of their clinical and immunological presentation may further explain how they contain the infection or progress to active TB. Additionally, the variances in the levels of IgA and IgG in patients, HHC and CC might be explained by the affinity maturation of activated B cells or isotope switching and generation of high affinity IgA and IgG (Abebe et al., 2018). In addition, we found a correlation between IgA serum and Covid-19 status of participants. As respiratory disease, both TB and Covid-19 can possibly produce IgA and study showed that LTBI and people with asymptomatic Covid-19 generated higher levels of IgA, IgG, IgM and neutralizing antibodies specific to SARS-CoV-2 (Rajamanickam et al., 2021). Prior exposure to *Mtb* might help in early recognition of SARS-CoV-2 peptides and activation and differentiation of memory T lymphocytes which results in strong humoral immune responses (Flores-Lovon et al., 2022).

The framework of protection of antibodies against TB is assumed to be varied and mostly reliant on the antigen specificity, isotypes, secretion sites, and route of administration (Tran et al., 2019). Our findings demonstrated that anti-HBHA IgA, and anti-LAM as well as anti-HBHA IgG responses are significantly higher both in LTBI and healthy individuals. By blocking the interaction with the mannose receptor on macrophages or binding to proteoglycans on the surface of the epithelial cells, the high affinity antibodies against *Mtb* antigens LAM and HBHA, respectively, could provide protection against the entry of *Mtb* within the host cells and development of stable infection. Anti-IgM HBHA has been reported

to prevent the cell adhesion of mycobacteria in a study (Shin et al., 2006) and LAM specific antibodies enhance both innate and cell-mediated immunity in the suppression of *Mtb* infection (Melkie et al., 2022). Therefore, the results of our study suggest that anti-HBHA IgA along with anti-LAM and anti-HBHA IgG could be the biomarkers of protective immunity. However, our data also demonstrated that anti-LAM IgA in saliva and serum did not have any significant difference among study participants. A possible interpretation could be that, as a mycobacterial glycolipid, LAM might not generate affinity maturation of B cells and class switching similar to protein antigens (Abebe et al., 2018).

Furthermore, we have additionally compared and observed the significant difference in IgA responses against LAM and HBHA in saliva and serum. In saliva, anti-LAM and anti-HBHA IgA levels were considerably greater in study participants (PTB, HHC, CC) compared to their antibody responses in serum (Fig: 6), indicating the importance of mucosal immunity against *Mtb* infection. In support of our view, significant immunological differences between the lungs and peripheral blood have been found in previous studies. A study by Schwander et al. (1996) showed bronchoalveolar cells responded more strongly to *Mtb* antigens and had a greater level of T lymphocyte activity in contrast to PBMCs (peripheral blood mononuclear cells) of active TB patients. When *Mtb* penetrates the host cells via mucosal surfaces (Abebe & Bjune, 2009), the interaction between host and bacilli does induce antibodies which are capable of binding *Mtb* antigens at the site of natural infection (Jacobs et al., 2016). Besides, anti-LAM and anti-HBHA IgA were also found to inhibit bacterial growth by human lung epithelial cells, suggesting the involvement of mucosal immunity against *Mtb* infection (Zimmermann et al., 2016). In addition, purified human secretory IgA (hsIgA) from colostrum of healthy women pre-incubated with *Mtb* in the lungs of mice was shown to minimize bacillary burden and improve disease progression (Alvarez et al., 2013, February).

We have used saliva in addition to serum as a study tool to measure IgA and IgG responses against *Mtb* antigens- LAM and HBHA. The saliva sample collection procedure is straightforward, non-invasive, reduces the risk of contamination to health personnel (Nahas et al., 2018) and is a beneficial tool to analyze mucosal immune responses against *Mtb* infection. SIgA is the most abundantly produced antibody in the upper airway (Li et al., 2020), and there is a clear indication that strong sIgA responses in saliva represent important aspects to support the role of mucosal immunity in protection and the choice of saliva as a promising tool to identify lung-specific protective mechanisms in the future. This has also provided important information about the route of vaccination. Macaques and humans' BAL samples exposed to

aerosol MVA85A exhibited higher levels of antigen-specific cellular immune responses (Morrison and McShane, 2021). Furthermore, intranasal administration of antigens together with mucosal adjuvant showed to stimulate sIgA formation (Li et al., 2012). The potential of mucosal vaccination to trigger immune response both in the lung mucosa and systemically is promising for vaccine design (Jacobs et al., 2016). In agreement with the above studies, we suggest that sIgA and mucosal immunity do play an interconnected role in protection and may have a significant implication for future vaccine development against TB. It has also highlighted the possibility that lung mycobacterial challenges and mucosal sampling (preferably saliva) may require more attention in the research of vaccine-induced immunological biomarkers of protection.

8. Methodological considerations:

8.1 Strengths:

This is the one of the few studies to measure and compare the IgA and IgG responses against LAM and HBHA in saliva. The study investigated the immunological parameters of antibodies both in serum and saliva against active and latency *Mtb* infection-associated antigens in naturally infected population in an endemic context. Noteworthy strength of the study is the structured questionnaire, inclusion and exclusion criteria which allowed to enroll the appropriate participants producing the reliable data. Furthermore, structured inclusion and exclusion criteria reduced selection bias and information biases were controlled by employing qualified health personnel and by providing the required training on standardized measurement.

8.2 Limitations:

The major limitation of the present study is the small number of sample size in cross-sectional study. The small sample size was not sufficient to provide statistical significance among some of the subgroups. Moreover, the generalizability of the study findings may be restricted to only the population in endemic settings. These data may thus constitute preliminary evidence of anti-LAM and anti-HBHA IgA and IgG responses among active TB patients, household contacts, and healthy controls. Thus, results need to be validated in a larger sample population.

Second, we have only explored the IgG response in the peripheral blood, not in saliva. Although some IgG is generated locally, the majority of IgG in saliva is sourced from serum, mostly through gingival crevices (Brandtzaeg, 2007). Since there may be a considerable degree of similarity between IgG responses in serum and saliva against *Mtb* antigens, we have solely evaluated IgG responses against LAM and HBHA in serum.

Lastly, we did not manage to successfully run IgA saliva and serum ELISA essay for some of the participants at the end of the study. The was due to resource limitation and time constraint to complete the study.

9. Conclusions:

TB is a complex disease and majority of the studies to date on host immune response to *Mtb* infection has been done on non-human primate models or in non-endemic settings. The present study investigated the sIgA and IgG responses against LAM and HBHA in human in an endemic region and highlighted the protective role of antibodies and involvement of mucosal immunity against *Mtb* infection. Our results, for the first time, showed the protective role of sIgA against HBHA of *Mtb* in saliva. The further findings demonstrated that the TB household contacts, and community controls compared to TB patients had significantly higher levels of IgA and IgG responses to LAM and HBHA in saliva and serum, confirming the role of antibodies in immune protection and reducing risk for progressing to active or acquisition of latent TB disease. Additionally, LTBI and healthy participants had higher levels of anti-HBHA IgA, anti-LAM and anti-HBHA IgG responses in serum in the present study, which indicates that these antibodies could be the biomarkers of protective immunity against TB. This study utilizes saliva as a study tool in addition to serum to measure IgA responses and observed that anti-LAM and anti-HBHA IgA levels in saliva were substantially higher in study participants than their antibody responses in serum, supporting the role of antigen-specific sIgA in protection and the significance of mucosal immunity in *Mtb* infection. Therefore, non-invasion sampling and the mucosal antibody-based TB vaccine candidates would also be of interest and could have a considerable impact for future vaccine development against TB.

9.1 Further research recommendations:

Considering the discussed limitations, further research would be more valuable. The cross-sectional study measures the exposure and outcome at a single point in time. For this reason, the implementation of a longitudinal study with a larger sample size would validate the result of IgA and IgG responses against *Mtb* antigens and give a sound conclusion. Therefore, a follow up investigation to the current study, new cohort would be suitable. Owing to varied antigen expression and distinct antibody isotypes at different phases of infections, the humoral immune response is diverse and complex (Melkie et al., 2022). Thus, a cohort study will enable to detect the *Mtb*-specific immunological markers associated to risk of progression to active TB and conversion to LTBI. In addition, the study design will also allow to identify the differences in the immune responses due to the different clinical stages of TB and measurement variation for the antibody isotype responses through follow-up. Population sampling prior to, or at multiple time points, and after treatment could provide a comprehensive knowledge of *Mtb*-specific mechanism of antibody responses and other immunological characteristics following treatment. Since the route of antigens' entry is one of the key determinants of antibody generation and class switching (Soe et al., 2021), for instance, the future study could also consider antibody isotypes in the mucosal lining (SIgA, IgM, or IgG) and investigate antibodies responses in saliva.

Moreover, the cross-sectional study does not allow to measure the frequency of exposure between the exposed and non-exposed study groups, which case-control study does. Therefore, a case control study could be conducted to identify, screen, and validate the potential of antigens against antibodies comparing active TB patient with healthy individuals in endemic and non-endemic context. This will minimize the confounders, such as, variation in infection pattern of index cases, duration and intensity of exposure and mutation of *Mtb* strains.

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Appendix I-English version

Consent form for tuberculosis patients

Title of the study: The protective role of secretory IgA and salivary IgG against *Mycobacterium tuberculosis* (*Mtb*) infection.

Background and purpose

A team of researchers is currently undertaking a study about protective immune responses against tuberculosis. Tuberculosis is one of the most important infectious disease that kills around 2 million people annually and causes illness in about 10 million people, globally. Ethiopia is one of the countries most affected with TB. The study aims to compare differences in IgA and IgG response to TB in patients (susceptible), household contacts (Mtb-exposed but apparently healthy), and community controls (with no history of TB/exposure and apparently healthy). Results of the study will form a basis for future design and development of an effective vaccine against TB.

You are invited to participate in the study because you have been diagnosed with pulmonary TB and you are about to start treatment.

What does the study bear?

If you agree to participate, you will be asked questions about TB disease, treatment, and possible transmission in your household. If you agree to participate, you will be interviewed about TB; you will undergo clinical and chest x-ray examinations; measurement of height and weight, your BCG vaccination status, and you will be asked to give blood (about 2 tea spoons) and saliva to measure antibodies. Participation in the study may incur minimal risks such as arm pain associated with needle prick during blood sample collection.

What happens to the information about you?

The information recorded about you will only be used for the purpose we have described above in accordance with the General Data Protection Regulation and Personal Data Act (The Personal Data Act (Lov 15. juni 2018 om behandling av personopplysninger and the General Data Protection Regulation (Regulation (EU) 2016/679) ("GDPR") of Norway. In Ethiopia,

the WMA Declaration of Helsinki-Ethical principles for medical research involving human subjects will be followed.

All data will be processed using serial number, instead of using your name or other recognizable information. The investigators will use private laptop for recording and storage of information. Only authorized personnel involved in the project will have access to your information. The information will be deleted once the project is completed, no later than 2026. It will not be possible to identify you from the results of the study and publications.

Voluntary Participation

It is voluntary to participate in this study. You can withdraw your consent to participate in the study at any time without giving any reason. This will not affect your future treatment. If you would like to participate, you will give your consent using your signature on the last page. If you agree now to participate, you can later withdraw your consent without any preconditions. If you want to withdraw from the study later or have questions concerning the study, you may contact the study team using the address described below.

Schedule

The study will take place starting Feb 2020 and may continue until 2021.

Possible side effects and inconvenience

The interview, clinical and chest x-ray examination may take some time (about an hour), and collection of blood and saliva may be inconvenient but will not cause health problems. If you encounter any health issue during these investigations, you will be treated immediately.

Possible benefits

You may not get direct benefits from the study. However, if the desired results leads to design and development an effective vaccine, your participation will save millions of people in Ethiopia and globally.

Participants' responsibility

You do not have any responsibility except providing information and biological materials required for the study. You will be informed as soon as possible if new information becomes

available that may affect your willingness to participate in the study. A token of appreciation (an amount equal to 100 and a soft drink) will be given to you to compensate the time lost or transportation cost, while participating in the study. In addition, if you spend money in connection with the study, you will be reimbursed.

Privacy

The information obtained about you will remain anonymous and confidential. There will not be a link with other registers. Only the investigators of the study have access to personally identifiable information.

Biobank

The biological samples (blood and saliva) will not be transferred to any other institution/ country. The anonymized samples will be destroyed after analysis at the Armauer Hansen Research Institute (AHRI).

Right of access and deletion of information about you

If you agree to participate in the study, you will be entitled to have access to information that is collected about you. You are also entitled to correct any inaccuracies in the information we have on you. If you withdraw from the study, all information collected about you will be deleted, unless the data is already entered in the analysis or used in scientific publications.

Economy

The study is entirely funded by the University of Oslo

Insurance

You will be given medical assistance if you become sick during the interview, and collection of blood and saliva.

Information on the outcome of the study

Anyone participating in the study will be informed of the results.

Declaration of consent

I have read and understood the information given above; I also understood the purpose and benefits of the research work. I am aware that participation in the study is absolutely voluntary and I can withdraw at any time during the course of the study without any consequences on me having assessed to health care services now or in the future. I am also aware that the information I give will be used strictly for research purposes and will be kept confidential. Finally, I declare that I have agreed to participate in the study in the presence of the witness.

Name of participant……signature---------- date----------

Name of a witness……. signature---------- date----------

Name of investigator…… signature---------- date----------

Note: the questions will be translated to Amharic, which is the local official language. A copy of the research protocol and support letter from Institute for Health and Society, University of Oslo will be sent to the Region for consent and approval.

Contact information

This study has received ethical approval from the AHRI/ALERT Ethics Review Committee and if you have any questions, please contact:

Rubiyat E-Eslam (University of Oslo, student researcher): Phone: +47 96755943

Dr. Fekadu Abebe (University of Oslo, Supervisor- retired) Phone: +47 40056237

Dr. Dominique Andree Yvette Caugant (Norwegian Institute of Public Health, Supervisor - Attending): DominiqueAndreeYvette.Caugant@fhi.no. Phone: +47 93863188.

or

Dr. Liya Wassie (Armauer Hansen Research Institute, Addis Ababa, Ethiopia, co-supervisor): Phone (0911664975) or

AHRI/ALERT Ethics Review Committee, Armauer Hansen Research Institute, Tel: +251 113 481289

Appendix II-Amharic version

ከቲቢ በሽተኞች ጋር ለሚኖሩ ወይም በአቅራቢያ ለሚኖሩ ተሳታፊዎች የተዘጋጀ ቅፅ

የጥናቱ ርዕስ፡ በሰውነት (ምራቅና ደም) ውስጥ ያሉ የቲቢ በሽታ መከላከያ ንጥረ ነገሮች (ኣይ ጂ ኤ እና የኣይ ጂ ጂ) ምርምር

የጥናቱ መግቢያና ዓላማ:

በኢትዮጵያና በኖርዌይ በሚገኙ የተለያዩ ተመራማሪዎች ቡድን በቲቢ በሽታ መከላከያ ዙሪያ ጥናትን ለማድረግ አቅደዋል፡፡ የቲቢ በሽታ በአለም ላይ በከፍተኛ ደረጃ ሞትን የሚያስከትል ሲሆን በአለም ላይ በየአመቱ 2 ሚሊዮን ሰዎችን የሚቀጥፍና ከ10 ሚሊዮን በላይ ለሚሆኑ ሰዎች ህመም ምክንያት በሽታ ነው፡፡ አገራችን በቲቢ በሽታ በከፍተኛ ደረጃ ከተጠቁ አገራት መካከል ናት፡፡ ይህ ጥናት በሰውነታችን ውስጥ በተለይም በምራቅና በደም ውስጥ ባሉ የቲቢ በሽታ መከላከያ ንጥረ ነገሮች (ኣይ ጂ ኤ እና የኣይ ጂ ጂ) ልኬት በቲቢ በሽታ በተጠቁ' ከቲቢ በሽተኛ ጋር አብረው በሚኖሩና ለቲቢ ተጋላጭነታቸው እምብዛም ባልሆኑ ሰዎች መካካል ያሉትን የንጥረ ነገር ልኬትን መመርመርና ማነፃፀር ሲሆን& በጥናቱ የሚገኘው ውጤትም ወደፊት ለሚደረጉ የቲቢ መከላከያ ክትባቶች ምርምሮችም ግብአትን ይሰጣል፡፡

እርስዎም በዚህ ጥናት ውስጥ ተሳታፊ እንዲሆኑ የተመረጡት ከቲቢ በሽታ ጋር ስለሚኖሩ ወይም በአካባቢው ስለሚኖሩ ነው፡፡

ጥናቱ ምን ያካትታል?

በዚህ ጥናት ውስጥ ፈቃደኛ ሆነው ከተሳተፉ ከቲቢ በሽታ ጋር ተያያዥ የሆኑ ጥያቄዎችን (ስለመድሃኒትዎ አወሳሰድ እና በቤትዎ ውስጥ ስላለው የበሽታው ስርጭት) እንጠይቅዎታለን:: በተጨማሪም በዚህ ጥናት ውስጥ ፈቃደኛ ሆነው ሲሳተፉ ስለቲቢ በሽታ ተጨማሪ ጥያቄዎችን እንጠይቅዎታለን@ የጤና ምርመራና የደረት ራጅ ምርመራ ይደረግሎታል@ እንዲሁም ቁመትዎ' ክብደትዎና በልጅነትዎ የወሰዱት የቢ ሲ ጂ ክትባት ምልክት ምርመራ ከተደረገልዎት በኋላ የደምና (2 የሻይ ማንኪያ የሚሆን) የምራቅ ናሙና እንዲሰጡ ይጠየቃሉ፡፡ በጥናቱ ውስጥ ሲሳተፉ ከደም ናሙና አወሳሰድ ጋር ተያይዞ መጠነኛ የሆነ ህመም በክንድዎ ላይ ሊሰማዎ ይችላል፡፡

ከእርስዎ የተወሰዱት ግላዊ መረጃዎችስ ምን ይደረጋሉ?

እንደ ‘General Data Protection Regulation and Personal Data Act, Lov 15. juni 2018 om behandling av personopplysninger and the General Data Protection Regulation (Regulation (EU) 2016/679)’ *መመሪያ መሰረት@* ለጥናቱ ተብሎ ከእርስዎ የተወሰዱ ግላዊና የህክምና መረጃዎችዎ በሙሉ ከጥናቱ ውጪ ለሆነ አላማ ፈፅሞ አይውሉም፡፡ በተጨማሪም ጥናቱ የአለም አቀፍ የህክምና ባለሞያዎች ማህበር ህግንና ደንብን ተከትሎ ይሰራል፡፡

ሁሉም ለጥናቱ ተብለው የተሰበሰቡት የግል መረጃዎችዎ በሚስጥራዊ ቁጥሮች ኮድ ይደረጋሉ@ የጥናቱ ተመራማሪዎችም መረጃዎችዎን በጥንቃቄ በኮምፒዩተር ላይ ቁልፍ ኮዶችን በመጠቀም ይይዛሉ፡፡ እነዚህም መረጃዎች ለጥናቱ ከተፈቀደላቸው የምርምሩ ቡድን አባላት በስተቀር ለሌሎች አይገለፅም፡፡ መረጃዎቹም ጥናቱ ከተጠናቀቀ በኋላ ይወገዳሉ፡ ፡ ከጥናቱ የሚወጡ መረጃዎችም ሆነ የምርምር ጥናት ፅሁፎች የእርስዎ ማንነት የማይገልፁ ናቸው፡፡

ፈቃደኛ ተሳትፎ

በዚህ ጥናት ውስጥ መሳተፍ በፍፁም ፈቃደኝነት ላይ የተመሰረተ ሲሆን@ በማንኛውም ጊዜ በጥናቱ ውስጥ ላለመሳተፍ ከፈለጉ ያለምንም ቅድመ ሁኔታ መውጣት ይችላሉ፡፡ ይህም ወደፊት የሚወስዱትን የህክምና አገልግሎት ሂደት አያጉላላውም፡፡ በጥናቱ ውስጥ ለመሳተፍ ፈቃደኛ ከሆኑ በዚህ ቅፅ የመጨረሻ ገፅ ላይ ስምዎንና ፊርማዎትን እንዲያኖሩ ይጠየቃሉ፡፡ በማንኛውም ሰዓት ከጥናቱ ለመውጣት ካሰቡ ካለምንም ቅድመ ሁኔታ ጥናቱን ማቋረጥ ወይም ከጥናቱ መውጣት ይችላሉ፡፡ ከጥናቱም ጋር ተያያዥ ጥያቄዎች ካሏችሁ ከስር በተጠቀሱት አድራሻዎች ሊያነጋግሩን ይችላሉ፡፡

የጥናቱ ጊዜያት

ይህ ጥናት እ.አ.አ. ከፌብሩዋሪ 2020 ጀምሮ እስከ 2021 ዓ.ም. ድረስ ይቀጥላል፡፡

ከጥናቱ ጋር ተያያዥ የሆኑ ስጋቶች

የጥናቱን መረጃዎች ከእርስዎ ለመሰብሰብ የምንጠቀማቸው ዘዴዎች እንደ ቃለ-ምልልስ' የጤናና የደረት ራጅ ምርመራ ሂደቶች እንዲሁም የደምና የምራቅ ናሙናዎች በሚሰበሰቡበት ጊዜ በተወሰነ መጠን የጊዜ መባከን ወይም በክንድዎ ላይ የመርፌ መጠነኛ የህመም ስሜቶች ሊያጋጥምዎ ይችላል፡፡ ይሁን እንጂ በጤናዎ ላይ የሚያደርሰው ጉዳት ወይም ስጋት የለም፡፡ በዚህ ሂደት ውስጥ ግን ምናልባት ከጥናቱ ጋር የተያያዘ የጤና እክል ቢፈጠር ወዲያውኑ የህክምና እርዳታ የሚያገኙ ይሆናል፡፡

የጥናቱ ሊገኙ የሚችሉ ጥቅሞች

በጥናቱ ውስጥ በመሳተፍዎ ቀጥተኛ የሆነ ጥቅም ላያገኙ ይችላሉ፡፡ ነገር ግን ከእርስዎ በተገኘው ናሙና ላይ ተመስርቶ የሚገኘው የምርምር ግኝት አመርቂ ውጤት ካስገኘ የቲቢ በሽታን ለመከላከል በሚደረገው አዲስ የቲቢ ክትባት ምርምር ላይ ተሳትፎ በማድረግዎ የብዙዎችን ህይወት ከቲቢ ለመከላከል ትልቅ አስተዋፅኦን ያደርጋሉ፡፡

የእርስዎ ሃላፊነት

በዚህ ጥናት ውስጥ ሲሳተፉ ትክክለኛ የጤና መረጃና የደም ናሙናን ከመስጠት በስተቀር የተለየ ሃላፊነት አይኖሮትም፡፡ ጥናቱ እየተካሄደ በዚህ ጥናት ውስጥ የመሳተፍዎን ሁኔታ ሊቀይር የሚችል የተለየ አዲስ መረጃ ከተገኘ እናሳውቆታለን፡፡ በጥናቱ ውስጥ በመሳተፍዎ ላባከኑት ጊዜ ወይም የትራንስፖርት ወጪ መጠነኛ የሆነ ክፍያ (በብር 100 እና የለስላሳ መጠጥ) ይሰጥዎታል@ በተጨማሪም ከጥናቱ ጋር ተያይዞ ያወጡት ወጪ ካለ ተመላሽ ይደረግሎታል፡፡

ሚስጥራዊነት

በጥናቱ ሂደት የሚሰበሰቡ የእርስዎ ግላዊ መረጃዎች በሙሉ ሚስጥራዊነታቸው ይጠበቃል፡፡ የሚሰበሰቡት መረጃዎችም ከሌላ የመረጃ ቋት ጋር አይገናኙም፡፡ እነዚህም መረጃዎች ለጥናቱ ከተፈቀደላቸው የምርምሩ ቡድን አባላት በስተቀር ለሌሎች አይገለፅም፡፡

የረጅም ጊዜ የናሙና ክምችት

ከእርስዎ የሚሰበሰቡት የደምና የምራቅ ናሙናዎች ወደ ሌላ ሃገር ወይም ተቋም አይተላለፉም፡፡ ለጥናቱ የተሰበሰቡት ናሙናዎች ጥናቱ ከተጠናቀቀ በኋላ በሙሉ ይወገዳሉ፡፡

የግል መረጃን ስለማግኘት ወይም ስለማጥፋት

በዚህ ጥናት ውስጥ ለመሳተፍ ከተስማሙ በጥናቱ ሂደት የሚሰበሰቡትን የራስዎን መረጃ ማግኘት ይችላሉ፡፡ በመረጃው ማሰባሰቢያ ወቅት ከሰጡት ውጪ ሆኖ የተሳሳተ ከሆነም ማረም ይችላሉ፡፡ ከጥናቱ ራስዎን ለማግለል ከፈለጉና ከእርስዎ የተሰበሰበው መረጃ ከመረጃ ማብላያ ወይም ከሳይንሳዊ ፅሁፎች ውጪ ከሆነ የተሰበሰበው መረጃ በሙሉ ይወገዳል፡፡

ይህ ጥናት ሙሉ በሙሉ የሚደገፈው በኦስሎ ዩኒቨርሲቲ ወጪ ነው፡፡

የመድህን ዋስትና

በጥናቱ ሂደት ውስጥ በጥናቱ ተሳትፎ ምክንያት የህክምና እርዳታ ቢያስፈልግዎ የህክምና እርዳታ ይደረግሎታል፡፡

የጥናቱ ውጤትን ስለማሳወቅ

በጥናቱ ሂደት ውስጥ የሚገለፁ አስፈላጊና ተገቢ የሆኑ የምርምር ውጤቶች ይገለፅሎታል፡፡

የስምምነት አንቀፅ

እኔ ከላይ የተገለፀውን የጥናት ቅፅ አንብቤ ወይም ተነቦልኝ ስለጥናቱ ተገቢውን መረጃ አግኝቻለሁ@ ገብቶኛልም፡፡ የጥናቱም አላማና ጥቅም ገብቶኛል@ በጥናቱ ውስጥ መሳተፍም በፍፁም ፈቃደኝነት ላይ የተመሰረተ እንደሆነና@ በማንኛውም ጊዜ ከጥናቱ ለመውጣት ወይም ላለመሳተፍ ብፈልግ ደግሞ አሁንም ሆነ ወደፊት የማገኘው የጤና አገልግሎት ሳይጓደል ጥናቱን ማቋረጥ እንደምችል ተገንዝቤአለሁ፡፡ በተጨማሪም ከጥናቱ ጋር ተያይዞ የተሰበሰበው መረጃ ለምርምሩ ስራ ብቻ የሚውል ሲሆን ሚስጥራዊነቱም እንደሚጠበቅ ተገንዝቤአለሁ፡፡ በመጨረሻም በጥናቱ ውስጥ ለመሳተፍ ፈቃደኛ መሆኔን በምስክር ፊት አረጋግጣለሁ፡፡

የጥናቱ ተሳታፊ ስም ,,, ፊርማ ,,, ቀን,,

የእማኝ/ምስክር ስም,, ፊርማ ,,, ቀን,,

የጥናቱ አስፈፃሚ ስም,, ፊርማ ,, ቀን,,,

ለተጨማሪ መረጃ

ይህ ጥናት በአህሪ/አለርት የምርምር ስነ-ምግባር ኮሚቴ ግምገማ የፀደቀ ሲሆን@ ከጥናቱ ጋር ተያያዥ ጥያቄዎች ካሎት በሚከተሉት አድራሻዎች በመደወል ማብራሪያ ማግኘት ይችላሉ፡፡

ሩቢያት ኢስላም (ከኦስሎ ዩኒቨርሲቲ፣ ተመራማሪ)፣ ስልክ ቁጥር +47 96755943

ዶ/ር ፈቃዱ አበበ (ከኦስሎ ዩኒቨርሲቲ፣ ዋና ሱፐርቫይዘር)፣ ስልክ ቁጥር +47 40056237

ዶ/ር ልያ ዋሴ (ከአህሪ፣ ሱፐርቫይዘር)፣ ስልክ ቁጥር 0911 664975 ወይም

የአህሪ/አለርት የምርምር ስነ-ምግባር ኮሚቴ፣ አርማወር ሃንሰን የምርምር ተቋም፣ ስልክ ቁጥር +251 113 481289

Appendix III-English version

Consent form for household contacts and community controls

Title of the study: The protective role of secretory IgA and salivary IgG against *Mycobacterium tuberculosis* (*Mtb*) infection.

Background and purpose

A team of researchers is currently undertaking a study about protective immune responses against tuberculosis. Tuberculosis is one of the most important infectious diseases that kills around 2 million people annually and causes illness in about 10 million people, globally. Ethiopia is one of the countries most affected with TB. The study aims to compare differences in IgA and IgG response to TB in patients (susceptible), household contacts (Mtb-exposed but apparently healthy), and community controls (with no history of TB or exposure to Mtb and apparently healthy). This will form a basis for future design and development of an effective vaccine against TB.

You are invited to participate in the study because you are sharing a household with pulmonary TB patients who are about to start treatment, or live in the neighborhood of TB patients.

What does the study bear?

If you agree to participate, you will be asked questions about your relationship with TB index cases and TB transmission in your household. If you agree to participate, you will be interviewed about TB; you will undergo clinical and chest x-ray examinations; measurement of height and weight and BCG vaccination status, and you will be asked to give blood (about 2 tea spoons) and saliva to measure antibodies. Participation in the study may incur minimal risks such as arm pain associated with needle prick during blood sample collection.

What happens to the information about you?

The information recorded about you will only be used for the purpose we have described above. We will process your personal data confidentially and in accordance with the General Data Protection Regulation and Personal Data Act (Lov 15. juni 2018 om behandling av personopplysninger and the General Data Protection Regulation (Regulation (EU) 2016/679)

("GDPR") of Norway. IN Ethiopia, the WMA Declaration of Helsinki-Ethical principles for medical research involving human subjects will be followed.

All data will be processed using serial number, instead of using your name or other recognizable information. The investigators will use private laptop for recording and storage of information. Only authorized personnel involved in the project will have access to your information. The information will be deleted once the project is completed, no later than 2026. It will not be possible to identify you with the results of the study and publications.

Voluntary Participation

It is voluntary to participate in this study. You can withdraw your consent to participate in the study at any time without giving any reason. This will not affect your future treatment. If you would like to participate, you will give your consent using your signature on the last page. If you agree now to participate, you can later withdraw your consent without affecting any preconditions. If you want to withdraw from the study later or have questions concerning the study, you may contact the study team using the address given at the end of this form.

Schedule

The study will take place starting Feb 2020 and may continue until 2021.

Possible side effects and inconvenience

The interview may take about 30 minutes of your time. In addition, you will be asked to undergo clinical and chest x-ray examinations, which may be inconvenient but carries no health risks. Experienced physicians and nurses under aseptic conditions will collect samples, but if you encounter any health issue during these investigations, you will be treated right away.

Possible benefits

You may not get direct benefits from the study. However, results of the study will be used to design and develop an effective vaccine that will save millions people in Ethiopia and globally.

Participants' responsibility

You do not have any responsibility except providing information and biological materials required for the study. You will be informed as soon as possible if new information becomes available that may affect your willingness to participate in the study. A token of appreciation (an amount equal to 100 and a soft drink) will be given to you to compensate the time lost and transportation cost, while participating in the study. In addition, if you spend money related to the study, you will be reimbursed.

Privacy

The information obtained about you will remain anonymous and confidential. There will not be a link with other registers. Only the investigators of the study have access to personally identifiable information.

Biobank

The biological samples (blood and saliva) will not be transferred to any other institution/country. The anonymized samples will be destroyed after analysis is completed at the Armauer Hansen Research Institute (AHRI).

Right of access and deletion of information about you

If you agree to participate in the study, you will be entitled to have access to which information that is collected about you. You are also entitled to correct any inaccuracies in the information we have on you. If you withdraw from the study, all the information about you will be deleted, unless the data is already entered in the analysis or used in scientific publications.

Economy

The study is entirely funded by the University of Oslo

Insurance

You will be given medical assistance if you become sick during the interview, and collection of blood and saliva.

Information on the outcome of the study

Anyone participating in the study will be informed of the results.

Declaration of consent

I have read and understood the information given above; I also understood the purpose and benefits of the research work. I am aware that participation in the study is absolutely voluntary and I can withdraw at any time during the course of the study without any consequences on me having assessed to health care services now or in the future. I am also aware that the information I give will be used strictly for research purposes and will be kept confidential. Finally, I declare that I have agreed to participate in the study in the presence of the witness.

Name of participant……signature---------- date----------

Name of a witness……. signature---------- date----------

Name of investigator…… signature---------- date----------

Note: the questions will be translated to Amharic, which is the local official language. A copy of the research protocol and support letter from Institute for Health and Society, University of Oslo will be sent to the Region for consent and approval.

Contact information

This study has received ethical approval from the AHRI/ALERT Ethics Review Committee and if you have any questions, please contact:

Rubiyat E-Eslam (University of Oslo, student researcher): rubiyati@uio.no; Phone: +47 96755943

Dr. Fekadu Abebe (University of Oslo, Supervisor- Retired): Fekadu.abebe@medisin.uio.no; Phone: +47 40056237.

Dr. Dominique Andree Yvette Caugant (Norwegian Institute of Public Health, Supervisor - Attending): DominiqueAndreeYvette.Caugant@fhi.no. Phone: +47 93863188.

Dr. Liya Wassie (Armauer Hansen Research Institute, Addis Ababa, Ethiopia, co-supervisor): (0911664975) or

AHRI/ALERT Ethics Review Committee, Armauer Hansen Research Institute, Tel: +251 113 481289

Appendix IV-Amharic version

ለቲቢ በሽተኞች የተዘጋጀ ቅፅ

የጥናቱ ርዕስ፡ በሰውነት (ምራቅና ደም) ውስጥ ያሉ የቲቢ በሽታ መከላከያ ንጥረ ነገሮች (ኣይ ጂ ኤ እና የኣይ ጂ ጂ) ምርምር

የጥናቱ መግቢያና ዓላማ:

በኢትዮጵያና በኖርዌይ በሚገኙ የተለያዩ ተመራማሪዎች ቡድን በቲቢ በሽታ መከላከያ ዙሪያ ጥናትን ለማድረግ አቅደዋል፡፡ የቲቢ በሽታ በአለም ላይ በከፍተኛ ደረጃ ሞትን የሚያስከትል ሲሆን በአለም ላይ በየአመቱ 2 ሚሊዮን ሰዎችን የሚቀጥፍና ከ10 ሚሊዮን በላይ ለሚሆኑ ሰዎች ህመም ምክንያት በሽታ ነው፡፡ አገራችን በቲቢ በሽታ በከፍተኛ ደረጃ ከተጠቁ አገራት መካከል ናት፡፡ ይህ ጥናት በሰውነታችን ውስጥ በተለይም በምራቅና በደም ውስጥ ባሉ የቲቢ በሽታ መከላከያ ንጥረ ነገሮች (ኣይ ጂ ኤ እና የኣይ ጂ ጂ) ልኬት በቲቢ በሽታ በተጠቁ' ከቲቢ በሽተኛ ጋር አብረው በሚኖሩና ለቲቢ ተጋላጭነታቸው እምብዛም ባልሆኑ ሰዎች መካካል ያሉትን የንጥረ ነገር ልኬትን መመርመርና ማነፃፀር ሲሆን& በጥናቱ የሚገኘው ውጤትም ወደፊት ለሚደረጉ የቲቢ መከላከያ ክትባቶች ምርምሮችም ግብአትን ይሰጣል፡፡

እርስዎም በዚህ ጥናት ውስጥ ተሳታፊ እንዲሆኑ የተመረጡት በቲቢ በሽታ የተጠቁና የቲቢ መከላከያ መድሃኒትዎን ለመጀመር በዝግጅት ላይ ስለሆኑ ነው፡፡

ጥናቱ ምን ያካትታል?

በዚህ ጥናት ውስጥ ፈቃደኛ ሆነው ከተሳተፉ ከቲቢ በሽታ ጋር ተያያዥ የሆኑ ጥያቄዎችን (ስለመድሃኒትዎ አወሳሰድ እና በቤትዎ ውስጥ ስላለው የበሽታው ስርጭት) እንጠይቅዎታለን:: በተጨማሪም በዚህ ጥናት ውስጥ ፈቃደኛ ሆነው ሲሳተፉ ስለቲቢ በሽታ ተጨማሪ ጥያቄዎችን እንጠይቅዎታለን@ የጤና ምርመራና የደረት ራጅ ምርመራ ይደረግሎታል@ እንዲሁም ቁመትዎ' ክብደትዎና በልጅነትዎ የወሰዱት የቢ ሲ ጂ ክትባት ምልክት ምርመራ ከተደረገልዎት በኋላ የደምና (2 የሻይ ማንኪያ የሚሆን) የምራቅ ናሙና እንዲሰጡ ይጠየቃሉ፡፡ በጥናቱ ውስጥ ሲሳተፉ ከደም ናሙና አወሳሰድ ጋር ተያይዞ መጠነኛ የሆነ ህመም በክንድዎ ላይ ሊሰማዎ ይችላል፡፡

ከእርስዎ የተወሰዱት ግላዊ መረጃዎችስ ምን ይደረጋሉ?

እንደ ‘General Data Protection Regulation and Personal Data Act, Lov 15. juni 2018 om behandling av personopplysninger and the General Data Protection Regulation (Regulation (EU) 2016/679)’ *መመሪያ መሰረት@* ለጥናቱ ተብሎ ከእርስዎ የተወሰዱ ግላዊና የህክምና መረጃዎችዎ በሙሉ ከጥናቱ ውጪ ለሆነ አላማ ፈፅሞ አይውሉም፡፡ በተጨማሪም ጥናቱ የአለም አቀፍ የህክምና ባለሞያዎች ማህበር ህግንና ደንብን ተከትሎ ይሰራል፡፡

ሁሉም ለጥናቱ ተብለው የተሰበሰቡት የግል መረጃዎችዎ በሚስጥራዊ ቁጥሮች ኮድ ይደረጋሉ@ የጥናቱ ተመራማሪዎችም መረጃዎችዎን በጥንቃቄ በኮምፒዩተር ላይ ቁልፍ ኮዶችን በመጠቀም ይይዛሉ፡፡ እነዚህም መረጃዎች ለጥናቱ ከተፈቀደላቸው የምርምሩ ቡድን አባላት በስተቀር ለሌሎች አይገለፅም፡፡ መረጃዎቹም ጥናቱ ከተጠናቀቀ በኋላ ይወገዳሉ፡ ፡ ከጥናቱ የሚወጡ መረጃዎችም ሆነ የምርምር ጥናት ፅሁፎች የእርስዎ ማንነት የማይገልፁ ናቸው፡፡

ፈቃደኛ ተሳትፎ

በዚህ ጥናት ውስጥ መሳተፍ በፍፁም ፈቃደኝነት ላይ የተመሰረተ ሲሆን@ በማንኛውም ጊዜ በጥናቱ ውስጥ ላለመሳተፍ ከፈለጉ ያለምንም ቅድመ ሁኔታ መውጣት ይችላሉ፡፡ ይህም ወደፊት የሚወስዱትን የህክምና አገልግሎት ሂደት አያጉላላውም፡፡ በጥናቱ ውስጥ ለመሳተፍ ፈቃደኛ ከሆኑ በዚህ ቅፅ የመጨረሻ ገፅ ላይ ስምዎንና ፊርማዎትን እንዲያኖሩ ይጠየቃሉ፡፡ በማንኛውም ሰዓት ከጥናቱ ለመውጣት ካሰቡ ካለምንም ቅድመ ሁኔታ ጥናቱን ማቋረጥ ወይም ከጥናቱ መውጣት ይችላሉ፡፡ ከጥናቱም ጋር ተያያዥ ጥያቄዎች ካሏችሁ ከስር በተጠቀሱት አድራሻዎች ሊያነጋግሩን ይችላሉ፡፡

የጥናቱ ጊዜያት

ይህ ጥናት እ.አ.አ. ከፌብሩዋሪ 2020 ጀምሮ እስከ 2021 ዓ.ም. ድረስ ይቀጥላል፡፡

ከጥናቱ ጋር ተያያዥ የሆኑ ስጋቶች

የጥናቱን መረጃዎች ከእርስዎ ለመሰብሰብ የምንጠቀማቸው ዘዴዎች እንደ ቃለ-ምልልስ' የጤናና የደረት ራጅ ምርመራ ሂደቶች እንዲሁም የደምና የምራቅ ናሙናዎች በሚሰበሰቡበት ጊዜ በተወሰነ መጠን የጊዜ መባከን ወይም በክንድዎ ላይ የመርፌ መጠነኛ የህመም ስሜቶች ሊያጋጥምዎ ይችላል፡፡ ይሁን እንጂ በጤናዎ ላይ የሚያደርሰው ጉዳት ወይም ስጋት የለም፡፡ በዚህ ሂደት ውስጥ ግን ምናልባት ከጥናቱ ጋር የተያያዘ የጤና እክል ቢፈጠር ወዲያውኑ የህክምና እርዳታ የሚያገኙ ይሆናል፡፡

የጥናቱ ሊገኙ የሚችሉ ጥቅሞች

በጥናቱ ውስጥ በመሳተፍዎ ቀጥተኛ የሆነ ጥቅም ላያገኙ ይችላሉ፡፡ ነገር ግን ከእርስዎ በተገኘው ናሙና ላይ ተመስርቶ የሚገኘው የምርምር ግኝት አመርቂ ውጤት ካስገኘ የቲቢ በሽታን ለመከላከል በሚደረገው አዲስ የቲቢ ክትባት ምርምር ላይ ተሳትፎ በማድረግዎ የብዙዎችን ህይወት ከቲቢ ለመከላከል ትልቅ አስተዋፅኦን ያደርጋሉ፡፡

የእርስዎ ሃላፊነት

በዚህ ጥናት ውስጥ ሲሳተፉ ትክክለኛ የጤና መረጃና የደም ናሙናን ከመስጠት በስተቀር የተለየ ሃላፊነት አይኖሮትም፡፡ ጥናቱ እየተካሄደ በዚህ ጥናት ውስጥ የመሳተፍዎን ሁኔታ ሊቀይር የሚችል የተለየ አዲስ መረጃ ከተገኘ እናሳውቆታለን፡፡ በጥናቱ ውስጥ በመሳተፍዎ ላባከኑት ጊዜ ወይም መጓጓዣ መጠነኛ የሆነ ክፍያ (በብር 100 እና የለስላሳ መጠጥ) ይሰጥዎታል@ በተጨማሪም ከጥናቱ ጋር ተያይዞ ያወጡት ወጪ ካለ ተመላሽ ይደረግሎታል፡፡

ሚስጥራዊነት

በጥናቱ ሂደት የሚሰበሰቡ የእርስዎ ግላዊ መረጃዎች በሙሉ ሚስጥራዊነታቸው ይጠበቃል፡፡ የሚሰበሰቡት መረጃዎችም ከሌላ የመረጃ ቋት ጋር አይገናኙም፡፡ እነዚህም መረጃዎች ለጥናቱ ከተፈቀደላቸው የምርምሩ ቡድን አባላት በስተቀር ለሌሎች አይገለፅም፡፡

የረጅም ጊዜ የናሙና ክምችት

ከእርስዎ የሚሰበሰቡት የደምና የምራቅ ናሙናዎች ወደ ሌላ ሃገር ወይም ተቋም አይተላለፉም፡፡ ለጥናቱ የተሰበሰቡት ናሙናዎች ጥናቱ ከተጠናቀቀ በኋላ በሙሉ ይወገዳሉ፡፡

የግል መረጃን ስለማግኘት ወይም ስለማጥፋት

በዚህ ጥናት ውስጥ ለመሳተፍ ከተስማሙ በጥናቱ ሂደት የሚሰበሰቡትን የራስዎን መረጃ ማግኘት ይችላሉ፡፡ በመረጃው ማሰባሰቢያ ወቅት ከሰጡት ውጪ ሆኖ የተሳሳተ ከሆነም ማረም ይችላሉ፡፡ ከጥናቱ ራስዎን ለማግለል ከፈለጉና ከእርስዎ የተሰበሰበው መረጃ ከመረጃ ማብላያ ወይም ከሳይንሳዊ ፅሁፎች ውጪ ከሆነ የተሰበሰበው መረጃ በሙሉ ይወገዳል፡፡

ይህ ጥናት ሙሉ በሙሉ የሚደገፈው በኦስሎ ዩኒቨርሲቲ ወጪ ነው፡፡

የመድህን ዋስትና

በጥናቱ ሂደት ውስጥ በጥናቱ ተሳትፎ ምክንያት የህክምና እርዳታ ቢያስፈልግዎ የህክምና እርዳታ ይደረግሎታል፡፡

የጥናቱ ውጤትን ስለማሳወቅ

በጥናቱ ሂደት ውስጥ የሚገለፁ አስፈላጊና ተገቢ የሆኑ የምርምር ውጤቶች ይገለፅሎታል፡፡**የስምምነት አንቀፅ**

እኔ ከላይ የተገለፀውን የጥናት ቅፅ አንብቤ ወይም ተነቦልኝ ስለጥናቱ ተገቢውን መረጃ አግኝቻለሁ@ ገብቶኛልም፡፡ የጥናቱም አላማና ጥቅም ገብቶኛል@ በጥናቱ ውስጥ መሳተፍም በፍፁም ፈቃደኝነት ላይ የተመሰረተ እንደሆነና@ በማንኛውም ጊዜ ከጥናቱ ለመውጣት ወይም ላለመሳተፍ ብፈልግ ደግሞ አሁንም ሆነ ወደፊት የማገኘው የጤና አገልግሎት ሳይጓደል ጥናቱን ማቋረጥ እንደምችል ተገንዝቤአለሁ፡፡ በተጨማሪም ከጥናቱ ጋር ተያይዞ የተሰበሰበው መረጃ ለምርምሩ ስራ ብቻ የሚውል ሲሆን ሚስጥራዊነቱም እንደሚጠበቅ ተገንዝቤአለሁ፡፡ በመጨረሻም በጥናቱ ውስጥ ለመሳተፍ ፈቃደኛ መሆኔን በምስክር ፊት አረጋግጣለሁ፡፡

የጥናቱ ተሳታፊ ስም ,,, ፊርማ ,,, ቀን,,

የእማኝ/ምስክር ስም,,, ፊርማ ,,, ቀን,,

የጥናቱ አስፈፃሚ ስም,,, ፊርማ ,,, ቀን,,

ለተጨማሪ መረጃ

ይህ ጥናት በአህሪ/አለርት የምርምር ስነ-ምግባር ኮሚቴ ግምገማ የፀደቀ ሲሆን@ ከጥናቱ ጋር ተያያዥ ጥያቄዎች ካሎት በሚከተሉት አድራሻዎች በመደወል ማብራሪያ ማግኘት ይችላሉ፡፡

ሩቢያት ኢስላም (ከኦስሎ ዩኒቨርሲቲ፣ ተመራማሪ)፣ ስልክ ቁጥር +47 96755943

ዶ/ር ፈቃዱ አበበ (ከኦስሎ ዩኒቨርሲቲ፣ ዋና ሱፐርቫይዘር)፣ ስልክ ቁጥር +47 40056237

ዶ/ር ልያ ዋሴ (ከአህሪ፣ ሱፐርቫይዘር)፣ ስልክ ቁጥር 0911 664975 ወይም

የአህሪ/አለርት የምርምር ስነ-ምግባር ኮሚቴ፣ አርማወር ሃንሰን የምርምር ተቋም፣ ስልክ ቁጥር +251 113 481289.

Appendix V

Questionnaire for assessment of socio-demographic characteristics, history of TB, clinical symptoms, and co-morbidities for TB patients.

S.No	Questions/ Variables	Coding category/response
1.	Name of Health Center	_____
2.	Study ID (SID)	_____
3.	Date of sample collection/Interview (DD/MMM/YYYY)	_____
4.	Age (in years)	_____
5.	Sex	☐Male ☐Female
6.	Marital status	☐Single ☐Married ☐Separated ☐Divorced ☐Widowed
7.	Education	☐ Not able to read and write ☐ Able to read and write without formal school year. ☐ Primary (1-8) ☐ Secondary (9-10 or 12) ☐ College (10+ or 12+) ☐ University degree

8.	Occupation	☐ Civil servant(gov't) ☐ Farmers ☐ Private worker ☐ Student ☐ Unemployed ☐Others (specify_____)
9.	Residence place	☐ With in Addis Ababa ☐ Outside of Addis Ababa
10.	Family size (members)	☐ 2 or less ☐ 3 - 5 ☐ 6 or more
11.	Relationship with contact	☐ Parents ☐ Siblings ☐ Spouse/ partner ☐ Son/Daughter ☐ Others
12.	BCG scar	☐ Present ☐ Absent ☐ Indeterminate
13.	HIV test result	☐ Positive ☐ Negative ☐ Not tested/don't know
14.	TB Testing	

	a. Have you ever been diagnosed or treated for Tuberculosis before?	☐Yes ☐ No ☐ Don't Know
	b. If 'Yes', when?	☐ Less than 2 years ago ☐ 2 years ago ☐ Between 2-5 yrs ago ☐ More than 5 years ago
	c. If you have been treated for TB before, did you complete your TB medications as your physician told to do?	☐Yes ☐ No
	d. Have you recently been tested for TB using a blood- or skin-based test?	☐Yes ☐ No ☐ Don't Know
	e. If 'Yes', which type of test, when tested and what was your result?	Which: _____ When: _____ Test result: _____
	f. Did you have a chest X-ray after the positive blood or skin test?	☐Yes ☐ No
	g. If 'Yes', what were the result of the x-ray?	☐ Normal ☐ Abnormal
15.	Clinical presentation	

	a. What TB symptoms do you currently have?	☐Cough more than 2 weeks ☐ Cough up blood or mucus ☐ Significant weight loss (4-5 kg in the last 2 months) ☐ Heavy night sweat (wetting bedsheet) ☐ Significant loss of appetite ☐ Fever
	b. Do you live with, or have you been in close contact with someone after you are diagnosed with TB (e.g., roommate, close friend, relative, family member)?	☐Yes ☐ No
16.	Have you been diagnosed or ill with COVID-19?	☐Yes ☐ No
17.	If 'Yes', when?	☐A year ago ☐ Recently
18.	Have you ever received immunosuppressive medications in the last 1 year?	☐Yes ☐ No
19.	If yes, for which disease(s)?	☐ Diabetics ☐ Cancer ☐ HIV ☐ Kidney ☐ Others ----------

Appendix VI

Questionnaire for assessment of socio-demographic data and risk for tuberculosis infection for household contacts and community controls.

General Information

S.No	Questions/ Variables	Coding category/response
1.	Name of Health Center	_____
2.	Study ID (SID)	_____
3.	Date of sample collection/Interview (DD/MMM/YYYY)	_____
4.	Age (in years) æ	_____
5.	Sex	☐Male ☐Female
6.	Marital status	☐Single ☐Married ☐Separated ☐Divorced ☐Widowed
7.	Education	☐ Not able to read and write ☐ Able to read and write without formal school year. ☐ Primary (1-8) ☐ Secondary (9-10 or 12) ☐ College (10+ or 12+)

		☐ University degree
8.	Occupation	☐ Civil servant(gov't) ☐ Farmers ☐ Private worker ☐ Student ☐ Unemployed ☐Others (specify_____)
9.	Residence place	☐ Within Addis Ababa ☐ Outside of Addis Ababa
10.	Are you pregnant? (For female participants only)	☐ Yes ☐ No ☐ Not tested
11.	BCG scar	☐ Present ☐ Absent ☐ Indeterminate
12.	HIV test result	☐ Positive ☐ Negative ☐ Not tested/don't know
13.	TB Screening	
	a. Have you ever been diagnosed or treated for Tuberculosis before?	☐Yes ☐ No ☐ Don't Know

	b. If 'Yes', when?	☐ Less than 2 years ago ☐ 2 years ago ☐ Between 2-5 yrs ago ☐ More than 5 years ago
	c. Have you recently been tested for TB using a blood- or skin-based test?	☐Yes ☐ No ☐ Don't Know
	d. If 'Yes', which type of test, when tested and what was your result?	Which: _____ When: _____ Test result: _____
	e. Have you had a chest X-ray in the last two years?	☐Yes ☐ No
	f. If 'Yes', what were the result of the x-ray?	☐ Normal ☐ Abnormal
	g. If 'Yes', from your Chest X-ray result, were you told that you had had scarring/fibrosis?	☐Yes ☐ No ☐ Don't know
14.	TB screening	
	a. Do you have the following TB-like symptoms currently?	☐Cough more than 2 weeks ☐ Cough up blood or mucus

		☐ Significant weight loss in the last 6 months ☐ Heavy night sweat (wetting bedsheet) ☐ Significant loss of appetite ☐ Fever
	b. Have you lived with, or been in close contact with someone who was recently diagnosed with TB (e.g., roommate, close friend, relative, family memeber)?	☐ Yes ☐ No
	c. If 'Yes', what is your family size (members)	☐ 2 or less ☐ 3 - 5 ☐ 6 or more
	d. Relationship with index case (TB patient)	☐ Parents ☐ Siblings ☐ Spouse/partner ☐ Son/Daughter ☐ Other
	e. How long did you live with the index case (TB patient)?	☐ Since birth ☐ < 3 months ☐ Between 3-6 months ☐ Between 6-12 months ☐ Between 1 - 2 years ☐ More than 2 years

15.	Have you been diagnosed or ill with COVID-19?	☐Yes ☐ No
16.	If 'Yes', when?	☐A year ago ☐ Recently
17.	Have you ever received immunosuppressive medications (not including inhaded steroids) in the last 1 year?	☐Yes ☐ No
18.	If yes, for which disease(s)?	☐ Diabetics ☐ Cancer ☐ HIV ☐ Kidney ☐ Others ----------
19.	Have you had any lumps in your neck, armpit or groin area which won't go away?	☐Yes ☐ No
20.	If 'Yes', have you been treated for the lumps? And when?	☐Yes ☐ No When: _____