Chapter 1

General Introduction

1.1 Introduction

1.1.1 Tuberculosis

Tuberculosis (TB) has been called a lot of names. The evangelist John Bunyan named tuberculosis 'the Captain of all these men of Death' and in India it was known as 'the King of Diseases' (69). It appears to be as old as humanity itself, with skeletal remains of prehistoric humans showing evidence consistent with TB of the spine. The early physicians called it *phthisis*, derived from the Greek term for wasting (113).

The World Health Organization reported 9 million new TB cases and about 2 million TB deaths for 2004. The number of TB cases for a few of the WHO regions was stable or decreasing, but in Africa the epidemic is still growing due to the spread of HIV. More than 80% of all TB patients live in sub-Saharan Africa and Asia. South Africa reports more TB cases than any other country in Africa (154).

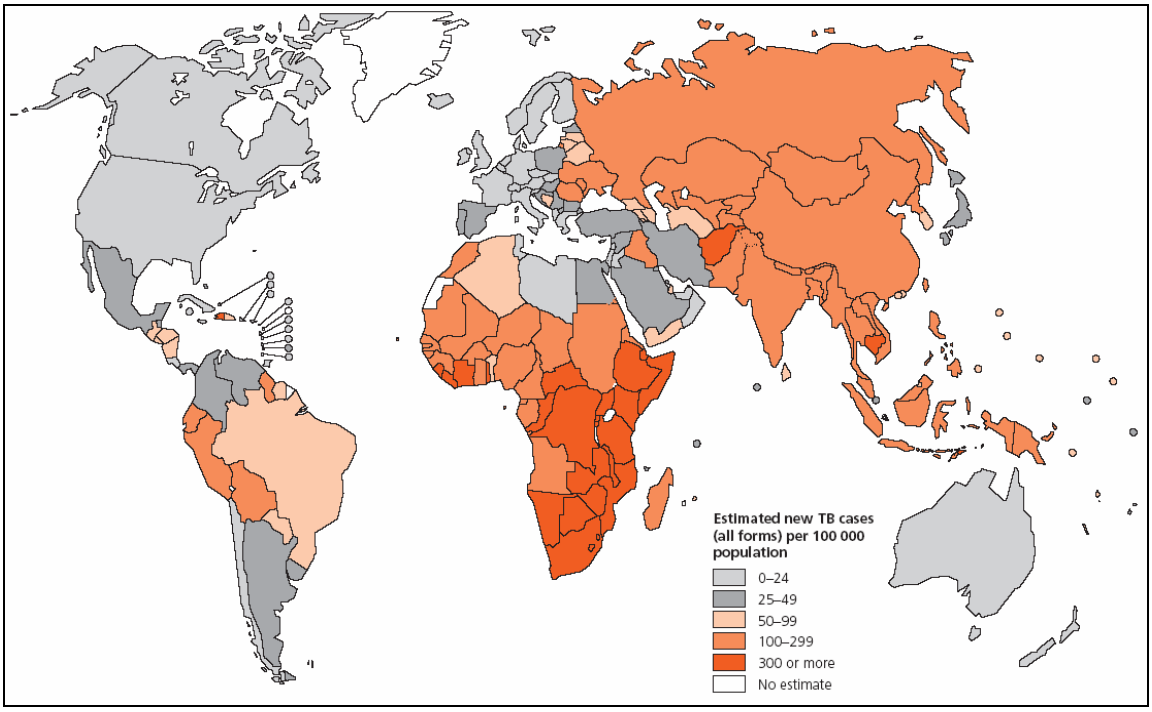

Figure 1.1: *Estimated TB incidence rates, 2004 (154).*

Among the 15 countries with the highest estimated TB incidence rates (Figure 1.1), 11 are in Africa. Incidence rates have been increasing since 1990, especially in African countries. The spread of HIV has driven the TB incidence higher in countries with high rates of HIV infection (154).

TB has been declared a global epidemic in the early 1990s. With the increase of multi-drug resistant (MDR) strains and the newly described extreme drug resistant (XDR) TB strain, the TB epidemic is still growing stronger. The need for quick diagnosis and better treatment of TB is now even bigger than before.

1.1.2 *Mycobacterium tuberculosis*, infection and immunology

In 1868, a French military surgeon, Jean-Antoine Villemin, experimentally demonstrated the transmissibility of tuberculosis in rabbits. It was considered that tuberculosis was likely to be caused by 'germs', and many workers attempted to isolate them. Then in 1882, the famous German Robert Koch, isolated and identified *Mycobacterium tuberculosis* (*M. tuberculosis*) as the causative agent for tuberculosis (69). *M. tuberculosis* is a rod-shaped, Gram positive, aerobic, acid-fast bacillus. The component responsible for acid-fastness is the unique lipid fraction of the cell wall, called mycolic acids. *M. tuberculosis* is an obligate pathogen, totally dependent on a living host for existence (54, 56, 113).

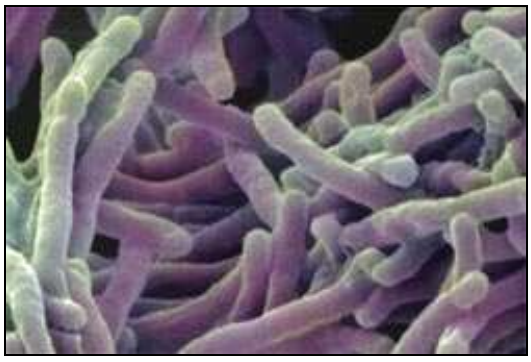

Figure 1.2: *M. tuberculosis.*

Individuals with TB spread *M. tuberculosis* through aerosolized infectious particles generated from coughing and sneezing. These bacilli enter via the respiratory route to infect mainly the lungs. From there, infection can spread through the lymphatic system or blood to other parts of the body (59, 117). After infection one of a few outcomes can occur: first, the bacilli can be destroyed immediately by the host's innate immune responses. Second, a percentage of persons

develop active tuberculosis (141). Third, the majority of people develop a clinical latent infection. These have a positive reaction to the skin test, but show no clinical symptoms and are not contagious (72, 138). About 5-10% of latent infections will reactivate and cause active tuberculosis (131).

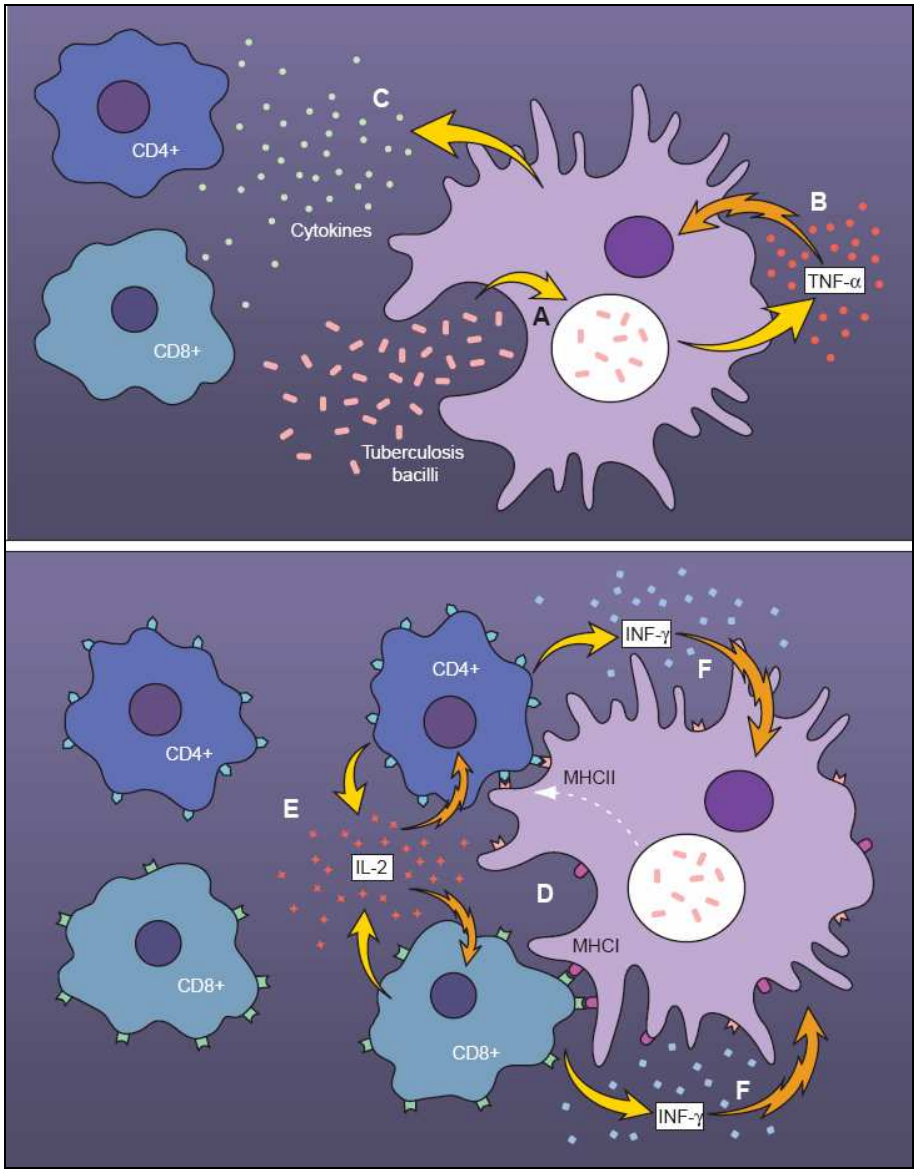

Figure 1.3: *The role of cytokines in TB. The macrophage (A) phagocytoses the invading mycobacteria, this results in the release of TNF-α (B) and other cytokines (C), the effect of which is further activation of cell-mediated immunity. The early release of TNF-α enhances the ability of macrophages to phagocytose and kill mycobacteria. Antigen presentation through MHC leads to the release (D) of interleukin-2 (IL-2) with further recruitment of T cells (E). T-cell release of interferon-γ (F) further activates the macrophage to enhance bacterial killing (94).*

Alveolar macrophages infected with *M. tuberculosis* interact with T lymphocytes via important cytokines. The T-cell response is antigen specific and is influenced by the major histocompatibility complexes (MHC) (60, 128). Infected macrophages release interleukins 12 and 18 which stimulate $CD4^+$ and $CD8^+$ T cells to secrete interferon-gamma (IFN-γ), which stimulates the phagocytosis of *M. tuberculosis* by the macrophage (55, 135). The macrophages also release tumour necrosis factor-alpha (TNF-α), which enables the macrophage to phagocytose and kill the mycobacteria (94) (Figure 1.3).

TNF-α plays a crucial role in the formation of granulomas and to control infection (20, 61, 107, 143). The granulomas isolate the infection and prevent their continued growth and spread to the rest of the lung and other organs and so concentrate the immune response to the site of infection (59).

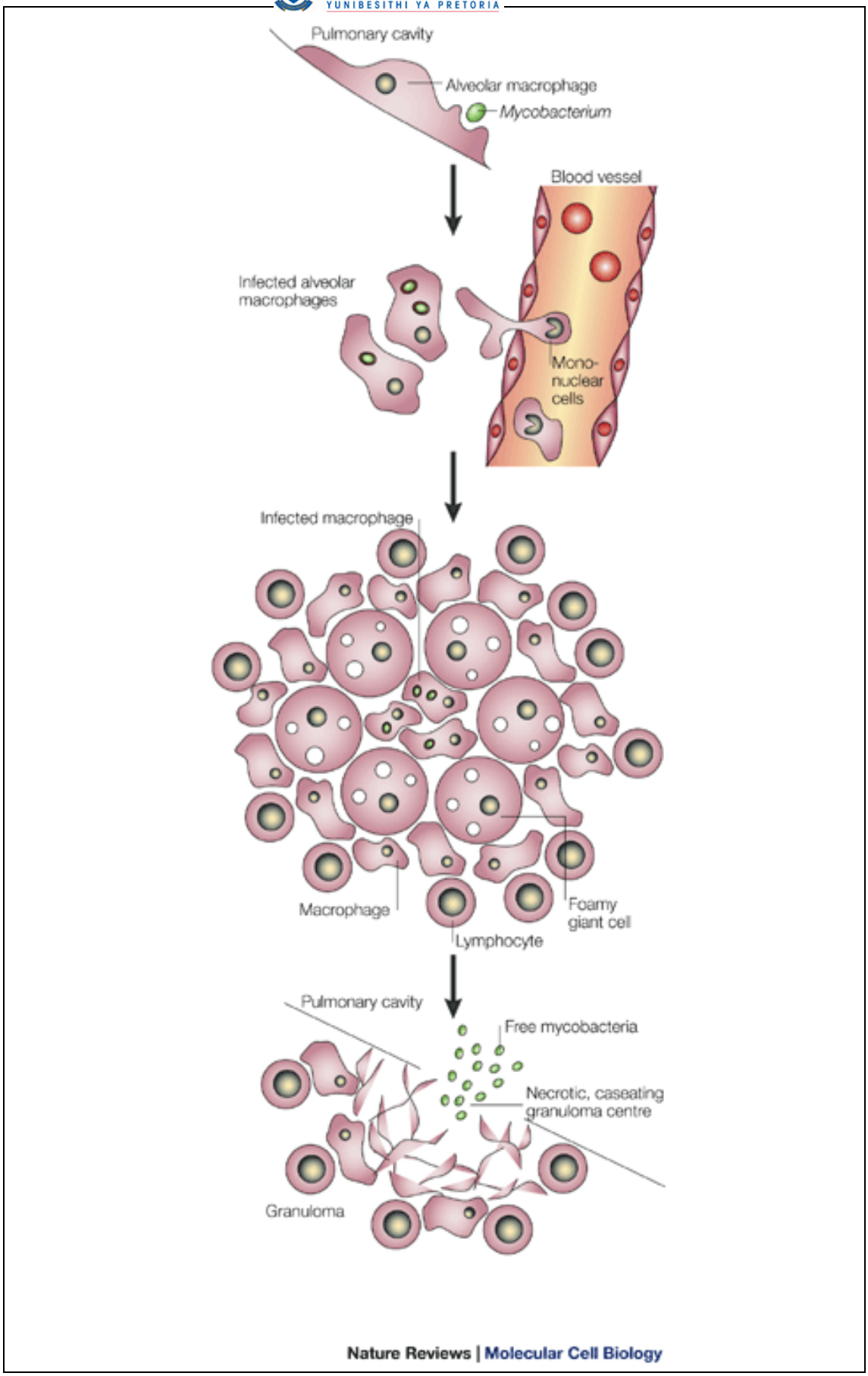

Figure 1.4: *Cellular events and granuloma formation following infection with* M. tuberculosis *(122).*

The success of granulomas depends on the number of macrophages present at the site of infection as well as on the number of organisms (56). The granuloma consists of a centre of infected macrophages surrounded by foamy giant cells, macrophages and lymphocytes (122). Figure 1.4 shows the cellular events and granuloma formation following infection.

The bacilli are unable to multiply within the granulomas due to a low pH and low availability of oxygen. With good host cell-mediated immunity, the infection may be stopped permanently at this point. The granulomas start to heal and leave small calcified lesions (137).

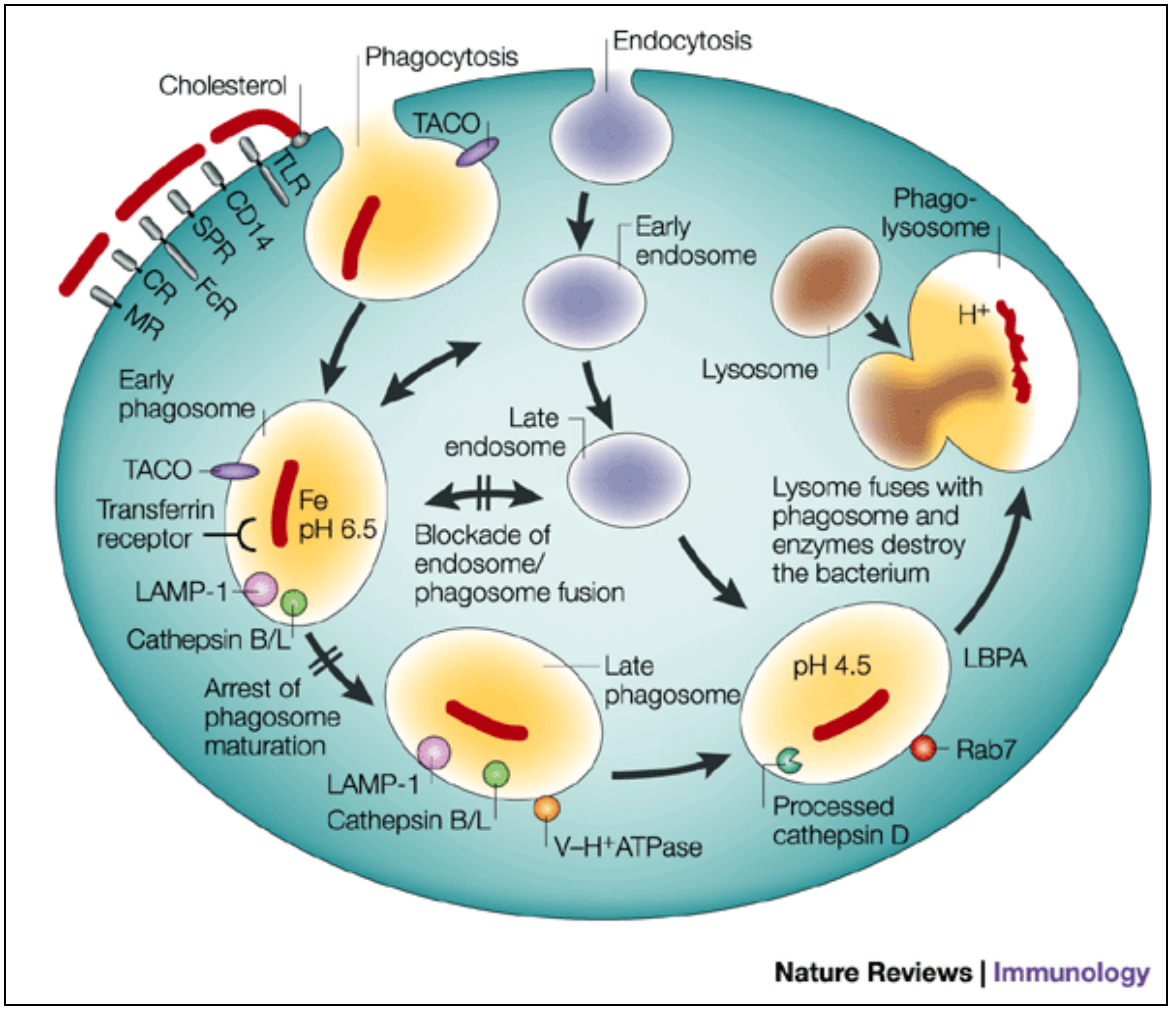

Figure 1.5: *The intracellular lifecycle of* M. tuberculosis. *CR, complement receptor; FcR, receptor for the constant fragment of immunoglobulin; LAMP-1 lysosomal-associated membrane protein 1; LBPA, lysobiphosphatic acid; MR, mannose receptor; Rab7, member of the small GTPase family; SPR, surfactant protein receptor; TACO, tryptophane aspartate-containing coat protein; TLR, Toll-like receptor; V–H$^+$ATPase, vacuolar ATP-dependent proton pump (83).*

Only a few organisms can survive inside the macrophage, due to hydrolytic enzymes and acidic phagocytic vacuoles. *M. tuberculosis* has immune evasive strategies to survive and multiply within the macrophage, probably because of the unusual mycobacterial cell surface (28). An important mechanism to the persistence and pathogenicity of mycobacteria in phagosomes is their ability to avoid fusion with lysosomes (73) that would destroy the bacteria. This process is dependent on living bacteria, as killed bacteria land up in the phago-lysosomes (13, 74).

Tryptophan-aspartate-containing coat protein (TACO) is absent from phagosomes containing killed bacilli, and from any of the endosomal/lysosomal organelles purified from uninfected cells. It was shown that TACO is retained by live mycobacteria at the mycobacterial phagosome membrane and that it prevents maturation and fusion with lysosomes, thus allowing the mycobacteria to survive inside the macrophage (2, 57), as shown in Figure 1.5.

1.1.3 Diagnosis of TB

Common clinical symptoms of TB infection include fever, night sweats, weight loss and shortness of breath, haemoptysis and chest pain. Laboratory tests for diagnosis of TB vary in specificity, sensitivity, speed and cost. Even though other tests are available, bacterial cell culture is still required for accurate diagnosis and drug-susceptibility testing (62). The other tests include the tuberculin skin test - of which the specificity is low and false positive and false negative reactions are frequent, direct microscopy - that is not adequately sensitive and requires a high level of bacteremia of more than 10 000 bacilli/ml sputum, ELISA to detect antibodies to *M. tuberculosis* - which is relatively simple and inexpensive, but most studies yielded poor sensitivity and specificity, chest X-rays - that can miss tuberculosis in HIV positive patients and cannot distinguish between lesions from current or past infections, and polymerase chain reaction (PCR) - that has created new possibilities for rapid and sensitive diagnosis, but still require a few days of culture (142).

1.1.4 Association with HIV/AIDS

TB is the leading cause of death amongst HIV infected people. According to WHO it accounts for about 13% of AIDS deaths worldwide and in Africa, HIV is the single most important factor playing a role in the increased incidence of TB in the past 10 years. TB and HIV together form a lethal combination (152). HIV prevalence in new TB cases in South Africa is 60% (Figure 1.6) (154).

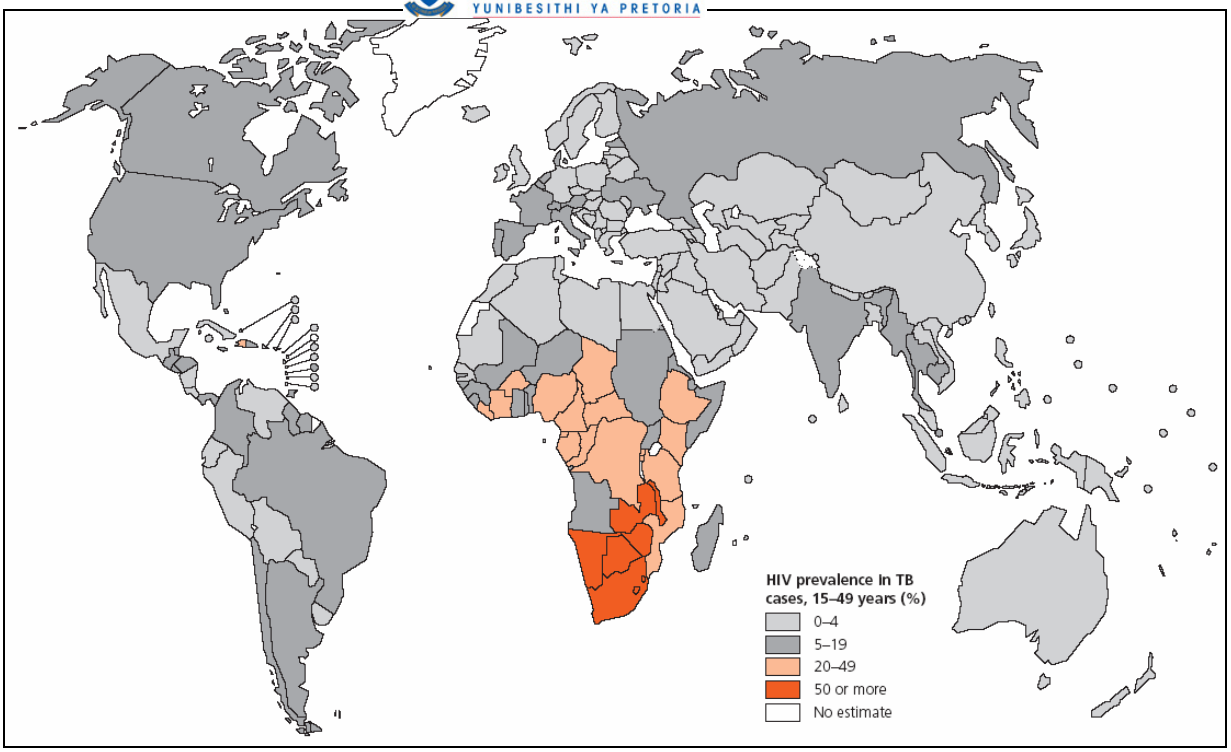

Figure 1.6: *Estimated HIV prevalence in new adult TB cases, 2004 (154).*

Human immunodeficiency virus (HIV) is a major risk factor for developing TB. In patients infected with HIV, the $CD4^+$ T cell count steadily decline as the infection progresses and the ability to restrict the bacilli to only a few infected macrophages decreases (111). Healthy people have about 1 000 $CD4^+$ T cells per microlitre of blood, which decline at a rate of about 100 cells per microlitre per year after HIV infection. A person usually develops TB at a count of approximately 187 $CD4^+$ T cells per microlitre of blood (156). The loss of T cell function reduces cytokine production, which compromises activation of macrophages. The activation of macrophages is required to control *M. tuberculosis* infection.

The risk of developing TB in patients co-infected with HIV and *M. tuberculosis* by reactivating latent tuberculosis infection increases to 5-15% annually, concurrent with the development of immune deficiency (12). In the 1993 classification of AIDS by the Centres for Disease Control, pulmonary TB was considered a true AIDS-defining illness in HIV-infected patients. It should be noted that there is a mutual interaction between *M. tuberculosis* and HIV: the immunosuppression induced by HIV infection, modifies the clinical presentation of TB resulting in atypical symptoms. Moreover, the immune restoration induced by highly active anti-retroviral therapy (HAART) may be associated with paradoxical worsening of TB manifestation related to immune reconstitution. TB, on the other hand, enhances the

progression of HIV infection, while anti-tuberculosis drugs interfere with anti-retroviral drugs (1).

In HIV negative patients infected with *M. tuberculosis*, clinical manifestations are pulmonary cavitation in the upper lobes and detectable bacilli in the sputum. With HIV infection this changes to extrapulmonary disease or infiltrating non-cavitating pulmonary disease in the lower lobes with no visible bacilli in sputum (111), which makes diagnosis of TB in HIV positive patients very difficult if not impossible. This strongly emphasises the need of a serodiagnostic test to accurately diagnose TB in a high HIV burdened population.

1.1.5 Diagnosis of TB in patients co-infected with HIV

Currently diagnosis of TB by growth of the Mycobacterium from sputum samples takes several weeks and is not always reliable (95), especially among HIV-infected communities. Different new methods for diagnosis are under investigation, of which the detection of antibodies in the sera of TB patients would be the most convenient and affordable (142). Protein and lipopolysaccharide antigens of TB are not good ligands for the antibodies, as antibody production to these antigens are paralysed by HIV co-infection, giving rise to false-negative results. This is not so with the mycolic acid antigens that make up the dominant part of the mycobacterial cell wall of *M. tuberculosis*. Tuberculosis patients produce antibodies directed against *M. tuberculosis* mycolic acids (112, 115). These antibodies were detected by an enzyme-linked immunosorbent assay (ELISA). This principle was reported by Schleicher *et al.* (127) to work especially well in HIV-co-infected patients, showing that HIV-infected patients were not disabled in any way to produce these antibodies in comparison to HIV-non-infected control, although the predictiveness of the test was still too low for practical application in diagnosis. The reason for this is that antibodies to the mycolic acids come about in a very different way in the body that is not affected by the human immunodeficiency virus (114, 133).

1.1.6 Other mycobacteria causing diseases

Besides *M. tuberculosis*, there are some other pathogenic mycobacteria as well, for example *Mycobacterium bovis, Mycobacterium africanum* and *Mycobacterium microti*, which are all related and members of the *M. tuberculosis* complex, (all members of *M. tuberculosis* complex cause tuberculosis). *M. bovis* is a causative agent of TB in many animals, the most significant being cattle, and some wildlife, like lions, buffalo and baboons which is an increasing problem

in the Kruger National Park (88, 99, 120). *M. microti* is a pathogen of voles and other small mammals, and *M. africanum* was isolated from man in equatorial Africa (69).

Then there are other mycobacteria including *Mycobacterium avium* complex (the avian tubercle bacillus), *Mycobacterium kansasii*, *Mycobacterium marinum* responsible for granulomatous skin disease of man, *Mycobacterium fortuitum* and *Mycobacterium chelonae* (69). Most of these mycobacteria cause TB-like diseases and opportunistic disease in immune deficient people, particular in AIDS patients (30).

There are also several non-tuberculous mycobacteria (NTM) that cause human diseases but not TB. *Mycobacterium avium paratuberculosis* has been identified as a possible cause of Crohn's disease in humans (129) and Johne's disease in animals. *Mycobacterium leprae* causes leprosy, which has declined to such an extent that it was eliminated from the list of global public health problems in 2000 (155). Buruli ulcer is a disease caused by *Mycobacterium ulcerans*, first described in Australia, which emerged in 1980 as an important cause of human suffering, making it the third most common mycobacterial infection after TB and leprosy (153).

1.1.7 Mycolic acids

1.1.7.1 History and properties of mycolic acid

An 'unsaponifiable' wax was first isolated from the human tubercle bacillus by Anderson and co-workers in 1938. He proposed to call this ether-soluble, unsaponifiable, high-molecular weight hydroxy acid 'mycolic acid'. Mycolic acid (MA) was described as an acid-fast, saturated acid with a low dextrorotation that contained hydroxyl and methoxy-groups. It was difficult to purify and impossible to crystallize. Pyrolytic cleavage yielded hexacosanoic acid and a non-volatile residue which showed no acidic properties. Together it showed an empirical formula of $C_{88}H_{172}O_4$ or $C_{88}H_{176}O_4$ (11, 140). In 1950, Asselineau proved that MAs contained a long alkyl branch in the α-position and a hydroxyl group in the β-position, thereby explaining the pyrolysis as a reversed Claisen reaction (14, 16). As the ability to elucidate the structure of such molecules advanced, the complexity of MA became more apparent. What was once described as a single component that was isolated from *M. tuberculosis* is now recognized as a family of over 500 related chemical structures. This amazing number of MA types that make up the major part of the cell wall of a single bacterium is an indication of the biological importance of these molecules (19).

Mycolic acids are very long, α-alkyl-β-hydroxy fatty acids present as mixtures of different types and homologues. All of the known MAs have the basic structure $R_2CH(OH)CH(R_1)COOH$ wherein R_1 is a C_{22} or C_{24} linear alkane and R_2, also known as the mero chain, is a more complex structure of 30-60 carbon atoms and contains a variety of functional groups (100, 101). Mycobacterial MAs are distinguished from those of the other genera by the following features: (i) they are the largest MAs; (ii) they have the largest side chain; (iii) they contain one or two unsaturated carbon moieties, which may be double bonds or cyclopropane rings; (iv) they contain oxygen functions additional to the α-hydroxy group; and (v) they have methyl branches in the main carbon backbone (101).

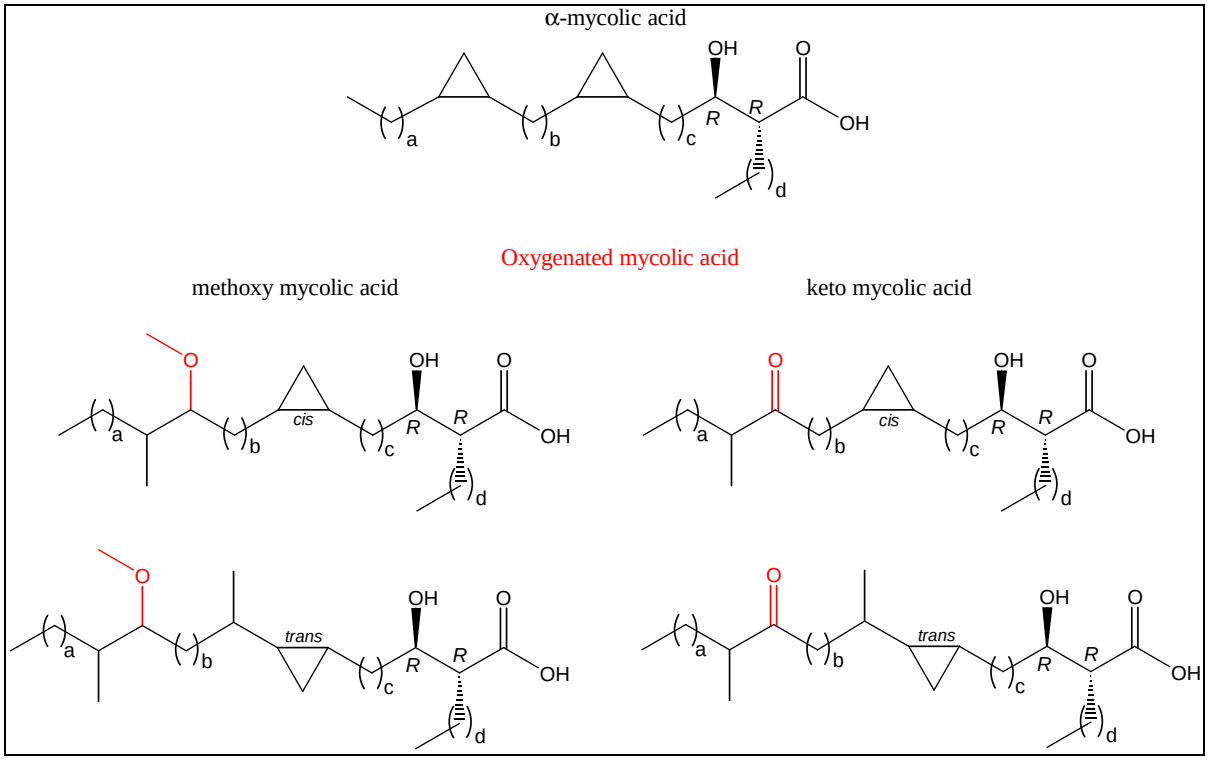

Figure 1.7: *Mycolic acid subclasses from* M. tuberculosis.

Mycolic acids from *M. tuberculosis* can be divided into three subclasses: α-MA, methoxy-MA and keto-MA (Figure 1.7). The prefix 'alpha' has been assigned to the acid lacking oxygen functions in addition to the 3-OH acid unit. The other mycolates are named according to the oxygen functions in the mero chain (15, 100, 101). α-Mycolic acid contains two *cis*-cyclopropanes, one in the proximal position (closer to the hydroxyacid) and one in the distal position (further away from the hydroxyacid) in the mero chain. Methoxy-mycolate has a *cis*- or *trans*-cyclopropane in the proximal position with a α-methyl-β-methoxy in the distal

position, whereas keto-MA also has a *cis*- or *trans*-cyclopropane in the proximal position, but with a α-methyl-β-keto in the distal position. When a cyclopropane ring is in the *trans*-orientation, it also contains an adjacent methyl branch (105, 106).

Mycolic acids show a variety of structural features in the meromycolate chain, including *cis*-cyclopropanes and α-methyl-*trans*-cyclopropanes with various combinations of α-methyl-β-keto, α-methyl-β-methoxy, *cis*-alkene, α-methyl-*trans*-alkene, and α-methyl-*trans*-epoxy fragments (100, 101). These acids are usually present as mixtures of various chain lengths. Although the hydroxyacid grouping is of the *R*,*R*-configuration, little is known about the absolute stereochemistry of the other groups. There is some evidence that the α-methyl-β-methoxy unit at the distal position from the hydroxyacid in MAs is of *S*,*S*-configuration (Figure 1.8) (44, 51).

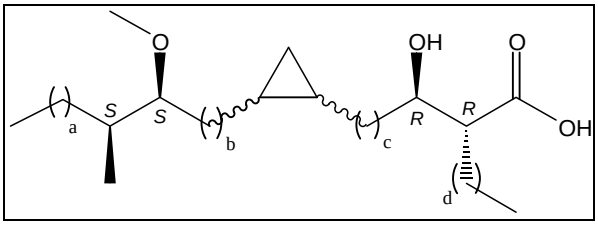

Figure 1.8: *The stereochemistry of the hydroxyacid group and the α-methyl-β-methoxy-group.*

Two-dimensional TLC (48, 104), GC (81) and HPLC (32, 116, 139) together with MS, IR and NMR techniques, made the identification of the different MAs present in each Mycobacterium possible. HPLC patterns are characteristic for each Mycobacterium and have been used as a rapid diagnostic tool (32).

Watanabe *et al.* (149, 150) isolated MA methyl esters from 24 strains of the *M. tuberculosis* complex and related mycobacteria. These were separated into the different subclasses and each subclass was further separated by argentation chromatography into mycolates with no double bonds, those with one *trans*-double bond and those with one *cis*-double bond. Mass spectrometry (MS) gave information about the chain lengths, while nuclear magnetic resonance (NMR) revealed the content of double bonds and cyclopropane rings. They further prepared mero MAs by pyrolysis of the methyl esters and submitted them to high-energy collision-induced dissociation/fast atom bombardment MS (CID/FAB MS) to reveal the exact location of the different functional groups within the merochain. These included double bonds,

cyclopropane rings, keto- and methoxy-groups. This revealed considerable variation in the mycolate composition. In the strains of the *M. tuberculosis* complex there are keto-, methoxy-, and α-MA that have a double bond in the proximal position in either the *cis*- or *trans*-configuration, instead of a cyclopropane ring. Watanabe *et al.* (149, 150) presented a possible classification of all these lipids. Type 1 defines the MAs with a cyclopropane (*cis*- or *trans*-), type 2 those with a *trans*-double bond and type 3 with a *cis*-double bond (149, 150) as shown in Figure 1.9. It was also shown that in several mero aldehyde homologues the values for a, b and c differed, with a and b more conserved than c.

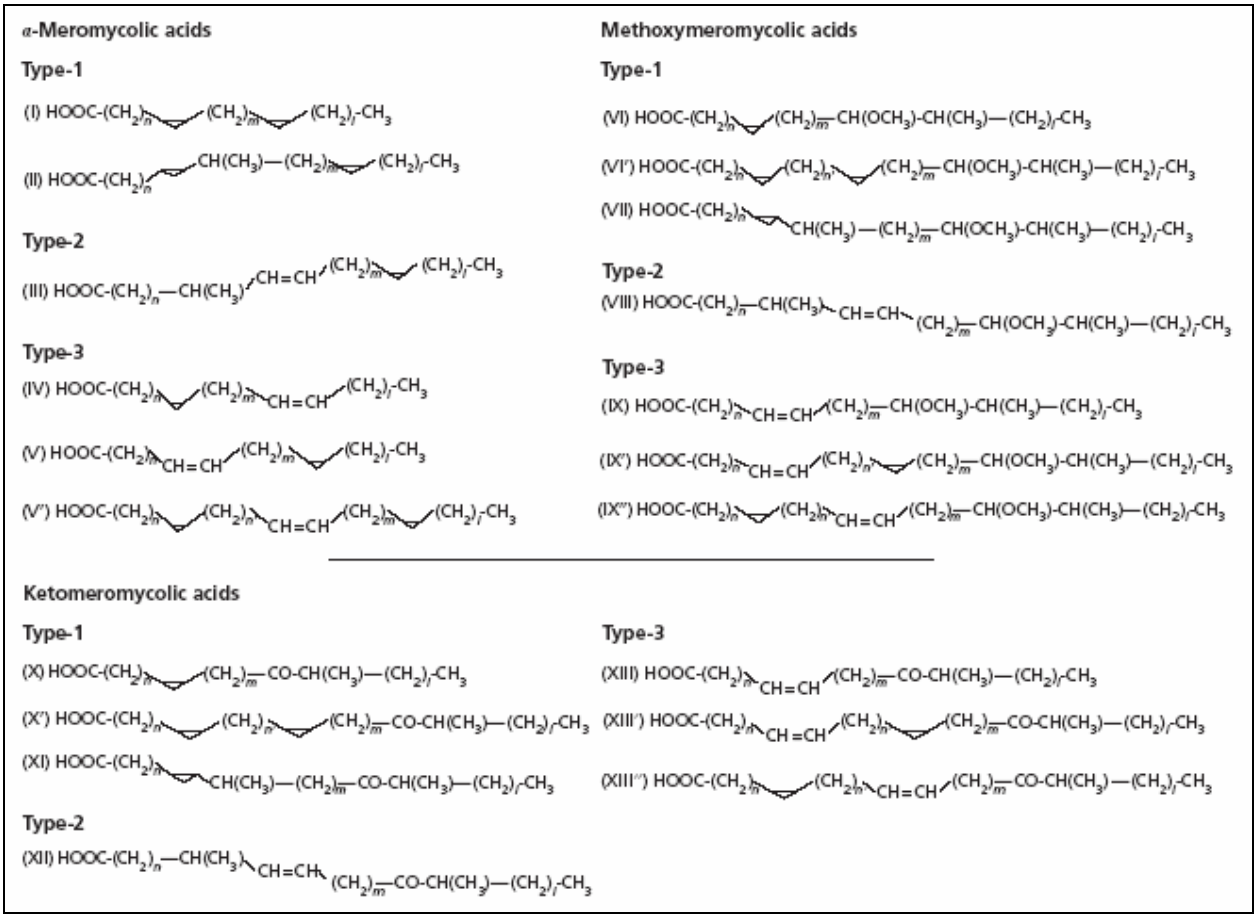

Figure 1.9: *Chemical structures for a-mero acids, methoxy-mero acids and keto-mero acids derived from MAs (149), n = c, m = b and l = a.*

Other mycobacteria contain different sets of MAs (Table 1.1 and Figure 1.10). For example, these can range from α'- and α- MA with either one or two double bonds, either in the *cis*- or the *trans*-configuration, and epoxy-MA in *M. smegmatis* and *M. fortuitum* (45, 71, 91, 103) to wax esters, ω-carboxy-MA and ω-1-methoxy-MA (92, 96) isolated from other mycobacteria.

A recent review confirmed that the most widespread of the different MAs is the α-mycolates, with either two cyclopropane rings or two double bonds (48, 148, 150). Keto-MAs are the second most widely distributed and are found in pathogenic and saprophytic mycobacteria. Wax esters and the α'-MA are next on the list.

Methoxy-MA are not frequently found in non-pathogenic mycobacteria (only about 12%), but are present in less than half of the mycobacteria that cause diseases in HIV co-infected individuals (Table 1.1)

Table 1.1: *Distribution of MA subclasses in several mycobacterial species (19)*

Species	α	α'	methoxy	Keto	epoxy	wax ester	ω-1 methoxy
M. abscessus	+	+					
M. africanum	+		+	+			
M. avium complex	+			+		+	
M. bovis	+		+	+			
M. chelonae	+	+					
M. fortuitum	+	+			+		+
M. gordonae	+		+	+			
M. intracellulare	+			+		+	
M. kansasii	+		+	+			
M. marinum	+		+	+			
M. scrofulaceum	+			+		+	
M. simiae	+	+		+			
M. tuberculosis	+		+	+			

Besides these major types of MAs, there are also other types present in mycobacteria; some in minor quantities. In 19 strains of the *M. tuberculosis* complex there are keto-, methoxy-, and α-MA which have a double bond in the proximal position either in the *cis*- or *trans*-configuration, instead of a cyclopropane ring (149, 150).

Although MAs are characteristic of all the mycobacteria, they are also present in genera other than *Mycobacterium*: in *Corynobacterium*, *Dietzia*, *Nocardia*, *Rhodococcus* and *Tsukamurella*, but all these bacteria have different kinds of MAs present in the cell envelope.

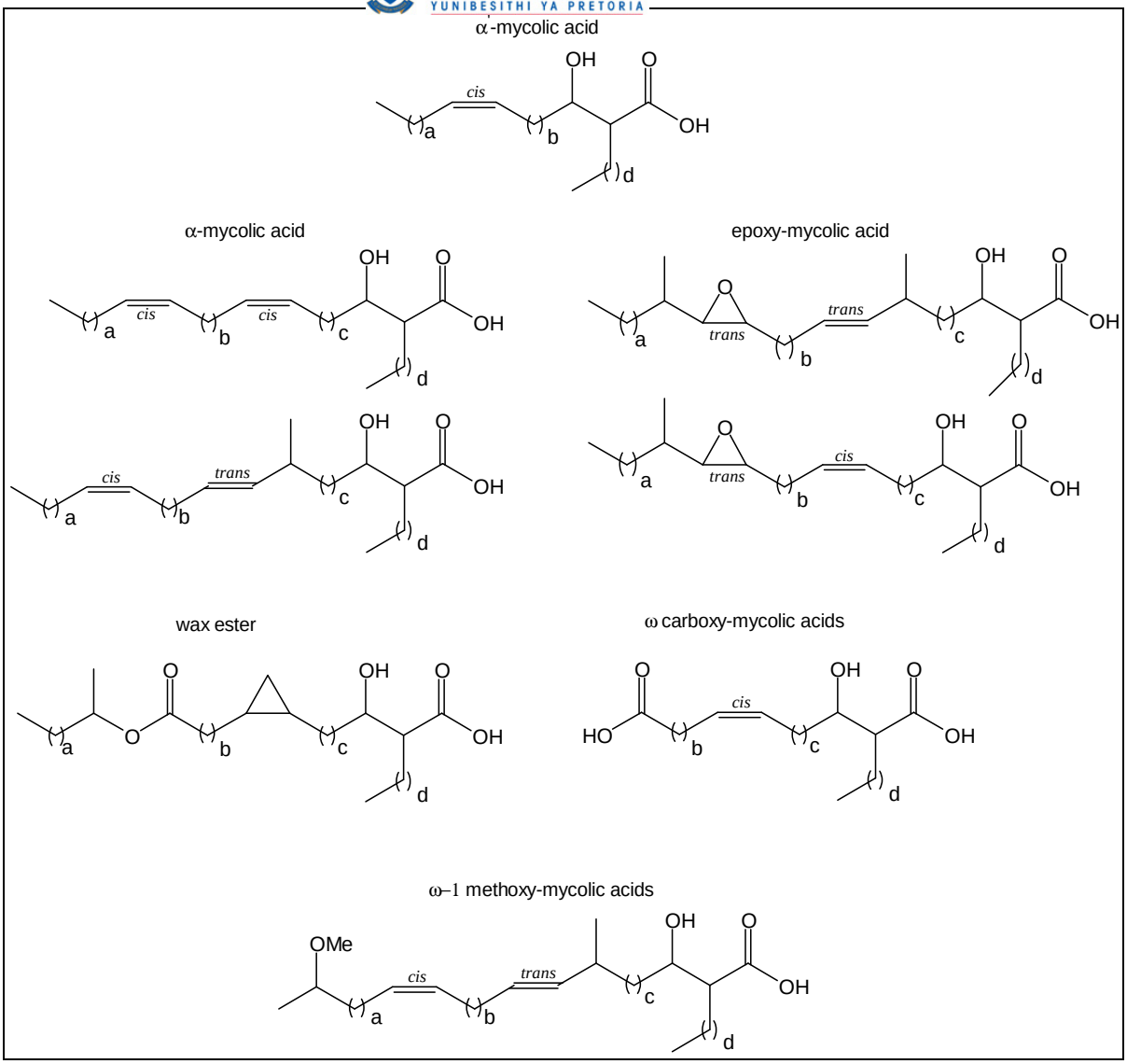

Figure 1.10: *Other MA subclasses found in* Mycobacterium spp.*, beside those in Figure 1.7.*

1.1.7.2 Mycolic acid and the cell wall

Mycolic acids are the major constituent of the cell envelope of *M. tuberculosis* and similar species, making up about 60% of the dry weight of the cell wall of the organism (30). Mycolic acids are present as organic solvent extractable lipids, mainly in the form of trehalose 6,6'-dimycolate also known as cord factor, but for the most part as bound esters of arabinogalactan, a peptidoglycan-linked polysaccharide (30, 100, 101).

The mycobacterial cell wall has a more complex structure compared to other Gram positive bacteria. The cell envelope consists of three structural components: the plasma membrane, the cell wall and the capsular layer (30, 42, 49), as shown in Figure 1.11. The mycobacterial cell wall is made up of three covalently linked macromolecules, namely peptidoglycan,

arabinogalactan and MA (29, 30, 98). Peptidoglycan is covalently attached to the mycolylarabinogalactan (mAG) through a phosphodiester bond. mAG, also called the cell wall core, constitutes the underlying framework of the cell wall and consists of MA covalently linked to arabinogalactan. The MAs are orientated perpendicular with respect to the plasma membrane. The C-2 branching position in the MA allows the meromycolate chain to pack closely in parallel with the saturated alkyl side chain. This packing is made possible by the flexibility of the arabinogalactan molecule and its linkage unit and is possibly stabilized by intramolecular hydrogen bonds between the MAs (100). Embedded into this framework is a uniquely large number of different lipids, several different multi-methyl branched fatty acids (25, 43, 100, 102), cell wall proteins, the phosphatidylinositol mannosides (PIMs), the phthiocerol-containing lipids, lipomannan (LM), lipoarabinomannans (LAM) (34, 35) and trehalose 6,6'-dimycolate (33).

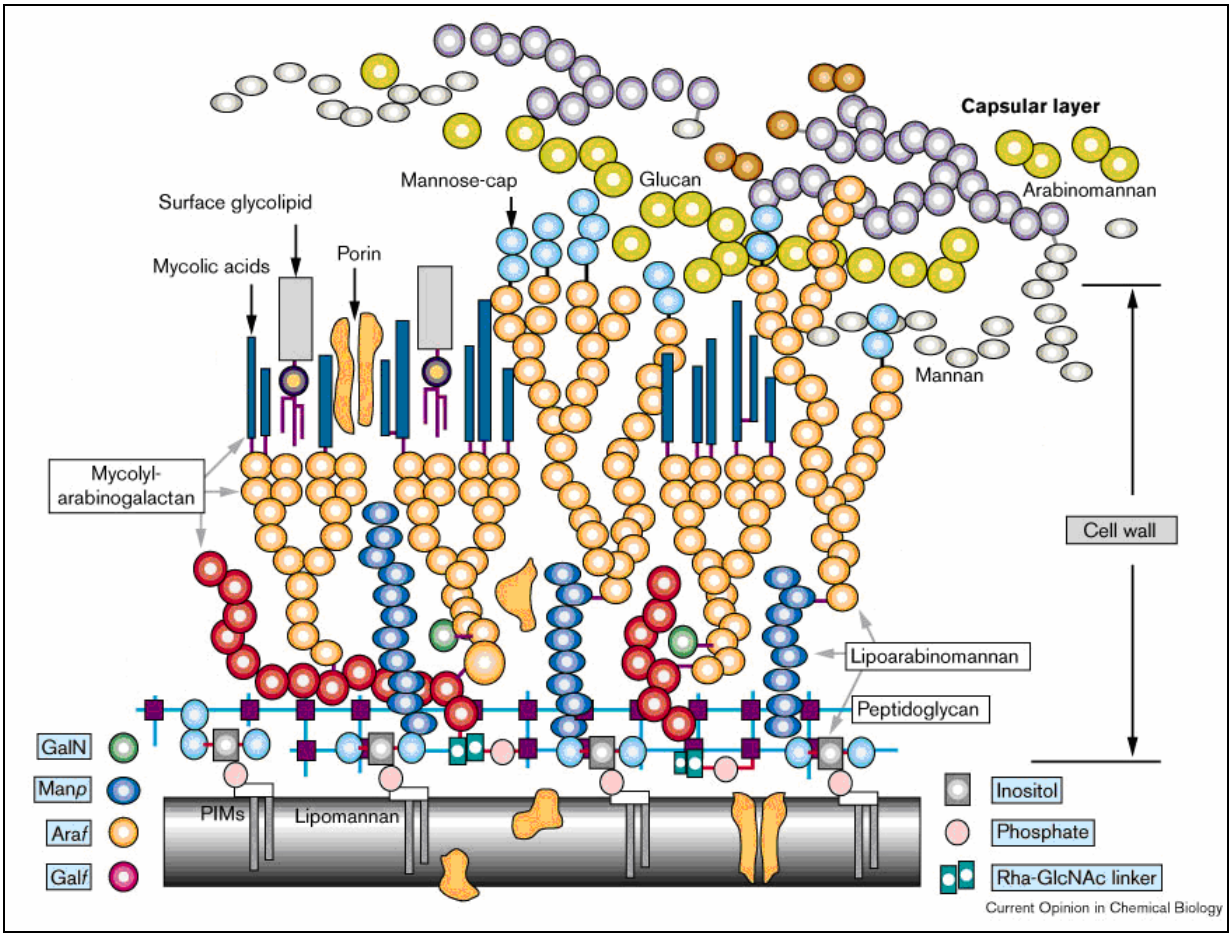

Figure 1.11: *A model of the mycobacterial cell wall (33).*

The cell wall, in particular MAs, is critical for growth and survival of *M. tuberculosis* in the infected host and they form an effective barrier against the penetration of antibiotics and chemotherapeutic agents (30). Some of the effective drugs are known to inhibit biosynthesis of the cell wall components. Mycobacterial pathogens are resistant to most common antibiotics and chemotherapeutics, and this might be due to the unusual structure and low permeability of the cell wall. Relatively hydrophobic antibiotics may be able to cross the cell wall by diffusing through the hydrophobic bilayer of MA and glycolipids, but hydrophilic antibiotics and nutrients cannot diffuse across this layer and make use of porin channels to cross (40, 136). There are various other lipids present in the mycobacterial cell envelope and these lipids may also take part in the permeability function of the cell envelope (100, 102). However, this study will focus on MA containing glycolipids.

1.1.7.3 Immunological properties of cord factor and mycolic acids

Trehalose 6,6'-dimycolate (cord factor) was discovered in 1950 by Bloch (27) and is an interesting glycolipid isolated from tubercle bacilli, a trehalose esterified at both primary alcohol groups with MAs (110), as shown in Figure 1.12.

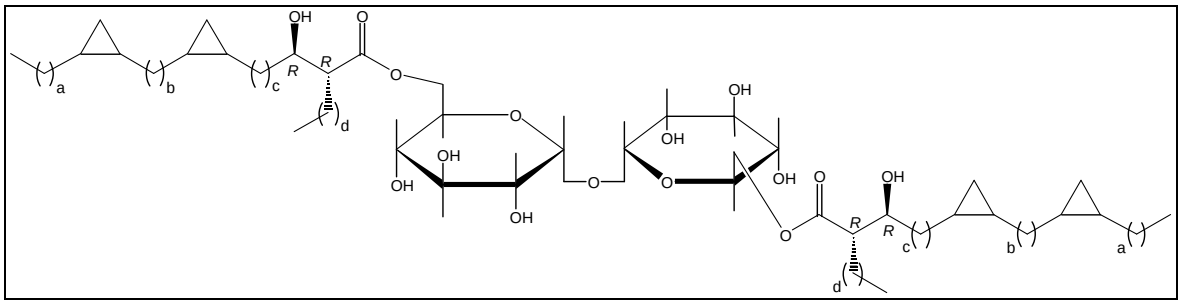

Figure 1.12: *Cord factor - trehahose esterified at both primary alcohol groups with MAs.*

Cord factor is considered a "free lipid" since it can be extracted from the cell wall, while the other MAs that are covalently linked to arabinogalactan cannot without hydrolysis. Cord factor has been shown to possess an array of immunological properties such as granuloma formation (18, 22), angiogenesis (125, 126) and anti-tumoral activity (23). It also protects against infection with other pathogenic micro-organisms and it is a potent adjuvant enhancing the immune system's response to diverse antigens (119, 123). Cord factor is important for the survival of *M. tuberculosis* in the macrophage as it appears to prevent fusion of phospholipid vesicles. Cord factor is stimulates innate, rather than adaptive immunity.

In 1971 Bekierkunst (23) indicated antibody production against cord factor, and in 1972, Kato speculated that it was the sugar part of cord factor that was recognised and not the lipid part (82). Recently a number of publications contradicted this as it was shown that the MA part may indeed be recognised (63, 64). It was reported that the detection of anti-cord factor IgG antibody by ELISA in active and inactive TB patients is a useful serodiagnostic tool and is applicable in various tuberculous diseases (78, 87, 97). In preliminary studies, Pan *et al.* (112) found that serum from tuberculosis patients were highly reactive against cord factor isolated from *M. tuberculosis*, whereas they were less reactive against cord factor from *Mycobacterium avium*. They also reported that *M. avium* patients' sera were highly reactive against *M. avium* cord factor, but less reactive against *M. tuberculosis* cord factor, thus suggesting that specific MA subclasses may be the antigenic epitope for the anti-cord factor antibody. They further demonstrated the antigenicity of MA by coating ELISA plate wells with the pure, separated MA methyl esters from hexane solutions and showed that among the three subclasses of MA present in *M. tuberculosis*, sera from TB patients reacted most prominently against the methoxy-MA and less against the α-MA and keto-MA. There was no reaction against either straight-chain fatty acid such as palmitic acid or shorter-chain MAs such as the C44-C46 nocardo-MAs.

Mycolic acids are extremely hydrophobic and dissimilar to other, more common antigens. The first evidence for immunoregulatory properties of MAs was obtained by the observation that presentation of MA to T-cells occurred by professional antigen-presenting cells (APC) in a major histocompatibility complex (MHC)-independent manner through CD1b molecules (21). It was also shown that MA were able to stimulate CD4, CD8-double negative (DN) T-cell proliferation (21, 67).

Other studies showed that immunoassays prepared with MA derivatives from *M. fortuitum* were highly effective for the serodiagnosis of TB. This organism also contains oxygenated MAs, but they are of a different subclass from the one present in *M. tuberculosis*. Therefore, some MA types are more helpful than others in the preparation of immunodiagnostic devices for TB detection (95).

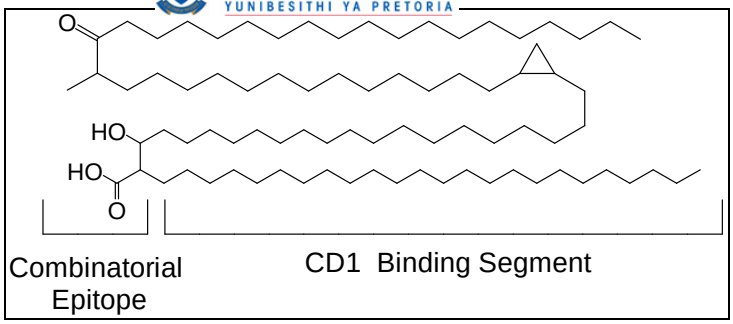

Figure 1.13: *A proposed folded conformation for keto-MA (70), (note that figure from refence is incorrect: the keto group and methyl branch are the wrong way around, the number of carbons between the hydroxyl group and the cyclopropane must be an odd number and those between the cyclopropane and the methyl-branched keto group must be an even number).*

More recently, Grant *et al.* (70) suggested that the keto-MA fold in a way that allows the three polar functions of the lipid chain to be in close proximity to form a combinatorial epitope presented on the CD1 surface protein (Figure 1.13), giving a possible reason for the stronger recognition of the oxygenated MAs. Hasegawa *et al.* (75, 76) also proposed that MAs could exist in folded conformations, although this was in the context of arrangement in monolayer films. These conformations are thought to be very stable in keto-MAs due to strong molecular interactive forces.

Recently, free MAs have been demonstrated to be able to adopt a “W” conformation with all four alkyl chains folded parallel to each other (130, 145, 146). The existence of this conformation has been suggested by Langmuir analyses of monolayers consisting of free MAs over a wide range of temperatures (145, 146).

1.1.7.4 Cholesterol in Tuberculosis

It appears that both the entry and the survival of the pathogen in the macrophage host cell depend on its attraction to cholesterol. Gatfield and Pieters (66) demonstrated that cholesterol concentrated at the specific site where mycobacteria enter the macrophage and that cholesterol depletion of the macrophage membrane prevented entry of the mycobacteria into the macrophage. This implies a mechanism of infection whereby the Mycobacterium, after docking to the macrophage, accrues cholesterol from the host membrane to the parasite cell wall and then penetrates the macrophage. The cholesterol enriched endosomic membrane then attracts and holds Tryptophan-Aspartate Coat (TACO) protein to prevent fusion of the infected organelle with the destructive lysosome (2, 58) (Figure 1.5). *M. tuberculosis* may facilitate its uptake into the host macrophage via the cholesterol binding Scavenger Type A receptor (159).

This molecular association between membrane cholesterol and mycobacterial MAs may also explain why the membrane of the phagosome appears to be tightly associated with the engulfed pathogen (46, 47). Av-gay and Sobouti (17) observed that pathogenic mycobacteria uniquely accumulates, but does not consume cholesterol from the growth medium, in contrast to the non-pathogenic mycobacteria that could rely on cholesterol as a major carbon source. Kaul *et al.* (85) discovered an infection-facilitating role for a "Human Receptor C_k-like" protein expressed in *M. tuberculosis*, of which the human equivalent is known for its function as a cholesterol sensor to regulate various genes for cholesterol homeostasis (84, 86). In addition, Korf *et al.* (89) showed that intraperitoneal MA administration into mice affectes mainly the macrophages and convert them into cholesterol-rich foam cells.

Mycolic acid can be considered as a likely candidate to attract cholesterol. It is the most abundant lipid of the outer layer of the mycobacterial cell wall. The “W” conformation of MAs could be imagined to resemble the shape of cholesterol; otherwise the structure of these two lipids upon first inspection appears to be very different. The waxy nature makes it highly hydrophobic, thereby making it possible to accumulate the hydrophobic cholesterol (30, 148). There is, however, much more involved than just hydrophobic interaction. In 2000, Dubnau *et al.* (50) demonstrated that recombinant *M. tuberculosis*, without the ability to oxygenate the MA side chains, could survive normally outside the macrophage in *in vitro* cell culture, but was unable to enter the macrophage. It could well be that an oxygenated group in the meromycolate chain is required for selective affinity towards binding cholesterol. This becomes an even likelier hypothesis by the discovery that the protein product of *M. tuberculosis’* inhA gene, an enzyme involved in the catalysis of the mero chain of the MA molecule, belongs to the family of the steroid dehydrogenases (121) and may therefore accommodate the meromycolate intermediates in its active site in a steroidal conformation.

1.2 Hypothesis

The recognition of MAs by TB patient serum antibodies, and the binding of MAs to cholesterol reside in only one of the subclasses of MAs that can be chemically synthesized and stereochemically characterized.

1.3 Aims

The aims of this study are to:

- Successfully separate natural mycolic acids into the different subclasses
- Determine antigenicity of the different subclasses
- Synthesize a stereochemically controlled methoxy-MA
- Determine the antigenicity of different types and diastereomers of synthetic mycolic acids
- Explore the binding of cholesterol to synthetic mycolic acids
- Determine structure-function relationships of mycolic acids