General Introduction
The World Health Organization (WHO) declared tuberculosis (TB) a global public health emergency in 1993. Although prevalence and death rates declined since then, TB remains one of the top 10 causes of death worldwide. In 2016, 10.4 million new TB cases were reported, of which 10% occurred in children and 10% in HIV-co-infected individuals (World Health Organization, 2017). TB is predominantly a pulmonary disease, but extra-pulmonary infection is particularly common in children under the age of 5 years and immune-compromised individuals (van Well et al., 2009). The most common form of central nervous system (CNS) involvement and most severe complication of TB is tuberculous meningitis (TBM). The incidence of TBM is estimated to be 1% of all TB cases, but varies greatly per location and is dependent on incidence of TB and HIV, age and living conditions (Wilkinson et al., 2017). Moreover, in TB endemic areas, TBM is the most common form of meningitis diagnosed in children (Rock et al., 2008). The incidence of TB and TBM seem to correlate directly, which means that strategies to reduce overall TB burden will eventually also improve the TBM problem (World Health Organization, 2017).

Infection with *Mycobacterium tuberculosis* leads to granuloma formation

*Mycobacterium tuberculosis* is transmitted via aerosols and consequently primarily infects lung tissue. In the lung, pathogenic mycobacteria are phagocytosed by alveolar macrophages. Subsequently, infected cells transport bacteria into deeper tissue where they give rise to granulomas (Pagan and Ramakrishnan, 2015; Ramakrishnan, 2012). Granulomas are the defining pathological hallmark of TB and are dynamic clusters of activated immune cells. The major function of granulomas is to prevent the spreading of bacteria in the surrounding tissue (Pagan and Ramakrishnan, 2015; Ramakrishnan, 2012). The predominant cell types that are recruited to early granulomas are macrophages, of which most cells differentiate into epithelioid cells, multi-nucleated giant cells or foamy macrophages (Flynn et al., 2011). Once a granuloma matures, other cell types, like neutrophils, natural killer cells and T and B-lymphocytes, are involved and the granuloma becomes surrounded by fibroblast and epithelial cells. The classic caseous granuloma is the most common type of granuloma in active and latent TB, in which dying macrophages and neutrophils form a necrotic core. The interface between this necrotic center and the surrounding epithelioid macrophage-rich region is the place where bacteria are most commonly found and grow out in higher numbers (Figure 1A, (Barry et al., 2009; Flynn et al., 2011; Mattila et al., 2013; Ramakrishnan, 2012). Additionally, mycobacteria can be found in epithelioid macrophages and in giant cells (Mattila et al., 2013). The periphery of the granuloma is formed by the lymphatic cuff, which predominantly contains T and B-lymphocytes and sporadic peripheral fibrosis. Other types of granuloma include non-necrotic, the predominant type in active TB, and fibrocalcific granulomas, seen mostly in latent TB (Figure 1B, C, (Barry et al., 2009; Mattila et al., 2013)). For long, granulomas were thought to be solely host protective structures exerting antimicrobial...
immune responses to restrict infection. However, recent work shows that mycobacteria are able to exploit granuloma formation for their survival, their local expansion and dissemination to new hosts, by manipulating host immune responses and cell death pathways (Davis and Ramakrishnan, 2009; Ramakrishnan, 2012).

In order to interact with host physiological mechanisms, *M. tuberculosis* has developed a uniquely complex mycobacterial membrane and strategies to secrete multiple...
virulence factors. To facilitate transport of virulence factors, pathogenic mycobacteria have up to five type VII secretion systems (T7SS). Of these systems, called ESX-1 to ESX-5, at least three are essential for virulence (Abdallah et al., 2007; Gröschel et al., 2016). ESX-1 is responsible for the secretion of the vital virulence factors ESAT-6 (EsxA) and CFP-10 (EsxB) and has been well characterized for phagolysosome escape after engulfment of mycobacteria by the macrophage (van der Wel et al., 2007). It has been well established that ESX-1 substrates play an essential role in phagosomal rupture (Houben et al., 2012; Simeone et al., 2012; van der Wel et al., 2007), although the exact molecular mechanism is still debated (Conrad et al., 2017; Peng and Sun, 2017). Subsequent bacterial entrance of the cytosol facilitates bacterial survival, but also triggers pro-inflammatory responses. Consequently, ESX-1 secretion has been associated with granuloma formation, recruitment of additional macrophages and dissemination of disease in addition to its role in the macrophage infection cycle (Carlsson et al., 2010; Gao et al., 2004; Stoop et al., 2011; Volkman et al., 2004).

**Rich focus theory**

Although *M. tuberculosis* primarily infects the lungs, it can spread to distant parts of the body. The current understanding of the pathophysiology of TBM is based on the pioneering work performed by Rich and McCordock in the 1920s and 1930s (Rich and McCordock, 1933). They were the first to challenge the long-standing hypothesis that TBM was a direct effect of hematogenous dissemination of *M. tuberculosis*. Instead they showed that lympho-hematogenous dissemination of *M. tuberculosis* from a primary pulmonary focus could lead to the formation of caseating foci in brain tissue, the so-called Rich foci. Bacterial proliferation can be contained within CNS granulomas and patients can remain clinically asymptomatic for many years post-infection. Ultimately, alterations in immune status or granuloma growth can lead to discharge of a substantial amount of bacteria into the subarachnoid space after which meningitis develops (Donald et al., 2005). Once a pre-existing Rich focus ruptures, the subsequent massive inflammatory response is responsible for the clinical manifestation of TBM. This can result in 1) additional tuberculoma formation, 2) basal inflammatory exudates causing obstruction of cerebral spinal fluid (CSF) and hydrocephalus and 3) obliterative vasculitis resulting in infarction (Figure 2, Be et al., 2009; Rich and McCordock, 1933; Wilkinson et al., 2017). Subsequent studies confirmed the observations made by Rich and McCordock and showed that different types of granulomatous lesions were found in all parts of the CNS; in the brain parenchyma, the meninges or both and adjacent to bone (Blacklock and Griffin, 1935; MacGregor and Green, 1935). The spectrum of lesions ranged from small (average 3 – 5 mm) and multiple caseous nodules (average of 30-40, range 1 - 130) to larger tuberculous exudative plaques (MacGregor and Green, 1935; Rich and Thomas, 1946). Granuloma can reach considerable size (6 cm) without produc-
ing meningeal inflammation if centrally located and can occur in absence of or along with TBM (MacGregor and Green, 1935; Rich and McCordock, 1933). The concept of the Rich focus still forms the center of thinking about the pathogenesis of TBM. However, a re-interpretation of this work suggests that in children miliary TB may have a stronger correlation with the onset of TBM than initially thought (Donald et al., 2005).

Distinctive patterns of granuloma formation are found in both histopathology as well as radiology studies (Bernaerts et al., 2003). There is clinical evidence to suggest that there is a varying response of the granuloma sub-types to treatment, which could suggest the differential outcome of clinical phenotypes observed at end of treatment.

**Figure 2. Schematic representation of Rich Focus theory**

After inhalation of *M. tuberculosis*, bacilli are phagocytosed by alveolar macrophages and transported into deeper tissue. Infected macrophages disseminate via the circulation and are able to cross the blood-brain barrier by a yet unknown mechanism. In brain tissue, Rich foci/granuloma are formed. Ultimately, alterations in immune status or granuloma growth can lead to discharge of a substantial amount of bacteria into the subarachnoid space, which lead to adhesions, vasculitis and infarction with subsequent clinical symptoms.

Adapted from (Be et al., 2009; Rich and McCordock, 1933; Wilkinson et al., 2017; van Well, personal communication).
Little is known regarding the specific clinical or histological aspects that may play a role in the formation of a granuloma, yet these characteristics can have important implications for the individual patient.

**Barriers between circulation and central nervous system**

In order to establish a site of infection within the brain parenchyma or meninges *M. tuberculosis* needs to cross the specialized barriers separating the CNS from the circulation. There are three barriers between blood circulation and brain tissue. The most widely studied barrier is the blood-brain barrier, which is present at all levels of the vascular tree (Figure 3A; Abbott et al., 2010, 2006; Obermeier et al., 2013). This barrier consists of specialized endothelial cells, which are connected by intercellular tight junctions, and tightly regulate movement of molecules. The BBB forms the dynamic and protective ‘neurovascular unit’ with several other CNS specific cell types, like neurons, pericytes, astrocytes and microglia (Abbott, 2013; Blanchette and Daneman, 2015; Hawkins and Davis, 2005). A second, less studied, barrier is the blood-cerebrospinal fluid barrier, which is formed by the epithelial cells of the choroid plexus (CP) that can be found in all ventricles of the brain (Figure 3B; Abbott et al., 2010; Lun et al., 2015). The CSF-facing epithelial cells are joined by tight junctions and are surrounding fenestrated capillaries and stromal tissue. The CP harbors pericytes closely connected to the endothelial cells and various immune cells. Moreover, the blood-CP barrier is thought to be an entrance point for immune cells into the CNS (Lun et al., 2015). The final barrier, the arachnoid barrier, is most likely not an important route for molecular or cellular entry into the CNS (Figure 3C; Abbott et al., 2010). This barrier is composed of multi-layered epithelium joined by tight junctions. Although this barrier is avascular, arachnoid villi connect with the circulation to aid reabsorption of CSF into the systemic circulation out of the brain in a one-way direction (Abbott et al., 2010). Little is known about how *M. tuberculosis* manages to cross the barriers protecting the CNS, but in theory pathogenic mycobacteria use at least one of the three known migration strategies: 1) transcellular migration, a receptor mediated strategy in which bacteria invade endothelial cells; 2) paracellular migration, achieved by disruption of the tight junctions and integrity of the BBB; or 3) the Trojan Horse mechanism, in which pathogenic bacteria utilize phagocytic cells as carrier to cross the BBB (Figure 4, Kim, 2008). The host and pathogen factors involved in migration of *M. tuberculosis* to the CNS have yet to be elucidated and will be discussed in this thesis.

**Immunological balance in mycobacterial pathogenesis**

Innate and adaptive immunological responses to mycobacterial infection are essential in combating the infection. Upon pathogen recognition, several cell signaling cascades lead to the production of pro-inflammatory cytokines, like tumor necrosis factor α,
TNFα (Flynn et al., 2011; van Crevel et al., 2002). This response will induce additional recruitment and activation of immune cells. Although this immune response is initiated to protect the organism it can also harm local neurons. Another key player in the CNS response against *M. tuberculosis* is vascular endothelial growth factor (VEGF). During TBM, increased VEGF levels have been found in CSF and are associated with vasculopathy, blood-brain barrier disruption, cerebral edema formation, hydrocephalus and basal meningeal enhancement (van der Flier et al., 2004; Visser et al., 2014). It is clear that an excessively active immune system can lead to tissue damage (Matty et al., 2015), suggesting that a balance between pro- and anti-inflammatory mechanisms is essential for a favorable outcome of disease (Flynn et al., 2011). The fact that Rich foci can be clinically asymptomatic for many years is probably a direct consequence and best example of this balance. Once the immune status is altered, a silent Rich focus can be reactivated and induce the massive inflammatory response seen during TBM. In addition, a very young age (<1 year) and advanced HIV-infection, are both associated with reduced protective immunity and are known for rapid TBM progression and high mortality (Wilkinson et al., 2017). This indicates that clinical features are influenced by the intracerebral inflammatory response.

![Figure 3. Blood-CNS barriers](image)

Schematic representation of the barriers between the CNS and circulation: [A] blood-brain barrier, [B] Blood-CSF barrier in the choroid plexus, [C] Arachnoid barrier. Image is combination of figures found in (Abbott et al., 2010, 2006; Lun et al., 2015; Obermeier et al., 2013).
Besides risk factors like age, HIV co-infection and malnutrition, genetic predisposition is also known to affect immune balance. Numerous genetic polymorphisms affecting the innate immune response to infection are associated with extra-pulmonary manifestation of TB. Links between Single Nucleotide Polymorphisms (SNPs) in host response pathways, including Toll-like receptors, with susceptibility to TBM are found in different ethnic groups (Caws et al., 2008; Dissanayeke et al., 2009; Hawn et al., 2006; Thuong et al., 2007; Wilkinson et al., 2017). An interesting example is a single nucleotide polymorphism (SNP) in the leukotriene A4 hydrolase ($LTA4H$) promoter. Depending on the specific genotype, this gene locus influences the balance between pro-inflammatory leukotriene B4 and anti-inflammatory lipoxin A4, with subsequent effect on TNFα levels (Tobin et al., 2013, 2010). The presence or absence of loci influencing cytokine levels and subsequent disease severity can have important implications for a single individual.

**Mycobacterial characteristics**

Next to the variation in the immune response we also have to consider the variation in the pathogen. Tuberculosis can be caused by any species belonging to the so-called *Mycobacterium tuberculosis* complex (MTBC). Detailed genetic analysis has shown that this complex encompasses seven lineages (lineage 1-7) that are adapted to humans. All these lineages are characterized by their own unique global distribution (Brites and Gagneux, 2017; Stucki et al., 2016), some are widespread and some are only identified in certain geographic areas. Specific *M. tuberculosis* lineages are thought to induce disseminated disease and TBM more frequently than others, although no mechanistic
explanation has been provided so far (Thwaites et al., 2008; Wilkinson et al., 2017). For instance, several studies in China suggest a predominance of Beijing isolates, belonging to lineage 2, in patients with extra-pulmonary TB and TBM (Pan et al., 2015; Xing et al., 2012). In Vietnam, it was shown that infection with lineage 2, in combination with a Thr597Cys polymorphism in the Toll-like receptor 2 (TLR2) gene, led to a higher risk on developing TBM (Caws et al., 2008). This suggests that the interplay between host and pathogen increases the risk for TBM. In a South African study among 285 children with culture proven tuberculosis no differences were found between the prevalence of lineage 2 and 4 in patients with TBM (Nicol et al., 2005). This probably means that both strains contribute equally to CNS involvement in this population.

With the help of transposon mutants and in vitro and in vivo work, specific genes necessary for CNS invasion have been studied. For example, studies in guinea pigs have revealed the importance of the gene *Rv0931c*, which encodes the serine/threonine protein kinase PknD, for invasion of brain endothelial cells (Be et al., 2012). However, whether this variation(s) in this gene is of clinical importance is not elucidated yet. An interesting gene identified in in vitro and in vivo studies to be of clinical importance is coding for PE_PGRS33. PE_PGRS33 is a secreted and cell surface localized protein shown to mediate entry of *M. tuberculosis* in macrophages through interaction with TLR2 (Palucci et al., 2016). A SNP in this gene was associated with an increased risk of developing TBM in Chinese children (Wang et al., 2011). Understanding the bacterial virulence factors involved in CNS invasion can help in improving our understanding of the pathogenesis of TBM. Moreover, these bacterial factors have a potential role as therapeutic target against TBM (Skerry et al., 2013).

**Clinical presentation of TBM**

In the majority of infected individuals, the initial immune response does not lead to complete clearance of infection, but often results in inhibition of mycobacterial outgrowth. Classically, this state, without eradication but also without replicating bacteria is called latent TB infection (LTBI) (Flynn et al., 2011) (Cadena et al., 2017). Globally, it is estimated that 2 billion people have a LTBI (Zumla et al., 2013) and that only 5-10% of this group develop active TB (World Health Organization, 2017). Although these definitions are still used in clinical practice, recent evidence support a spectrum of infection outcomes within these two states with considerable host and bacterial heterogeneity and varying symptoms (Cadena et al., 2017). Patients with sterilized or well-contained pulmonary infection are probably not at risk to experience reactivation of infection, while the group of patients with low-grade subclinical LTBI has a considerable higher risk of progression into active disease. Additionally, sterile granulomas and granulomas with active disease can be present at the same time in a single individual (Cadena et al., 2017; Flynn et al., 2011). Risk factors for reactivation or fast progression into active TB infection include
age, malnutrition and smoking. In addition, diseases affecting the immune response, like HIV co-infection, contribute to this risk (Wilkinson et al., 2017).

Despite extensive research efforts, clinical practice still faces many challenges in managing the burden of TBM. Diagnosis and treatment are often delayed due to the aspecific symptoms during the early stages of disease and suboptimal diagnostic tools, while accurate and fast start of treatment is thought to be the most important factor determining outcome (Mai and Thwaites, 2016; Thwaites et al., 2013). TBM stage I usually starts insidiously in infants, with aspecific symptoms like poor weight gain, low-grade fever and general illness. The most prominent factors that differentiate between TBM and common illnesses like influenza is duration of symptoms for more than 5 days and poor weight gain for weeks to months before presentation in TB endemic areas. Nevertheless, symptom persistence often goes unnoticed when patients are examined by different health care professionals (Bahr et al., 2016; Thwaites et al., 2013; van Toorn et al., 2012; van Well et al., 2009). As the disease progresses from TBM stage II to III, the most advanced stage of disease, neck stiffness is almost always present (van Well et al., 2009) and loss of consciousness, motor paresis and convulsions will follow. A common complication of TBM is hydrocephalus and is, together with many other symptoms like cranial nerve palsy, brainstem dysfunction, infarctions, and convulsions, associated with a poor outcome (van Toorn et al., 2012; van Well et al., 2009). In nearly half of the patients diagnosed, TBM is often only considered in the most advanced stage of the disease. This results in a high mortality rate of 20% and development of neurological sequelae in at least half of TBM survivors (Chiang et al., 2014; Mai and Thwaites, 2016).

**Diagnostic approach**

Currently, there is no single diagnostic method that is both sufficiently rapid and sensitive (Bahr et al., 2016). Extremely supportive in early diagnosis of TBM is neuro-radiological imaging. The common three radiological findings in TBM are: 1) Basal meningeal enhancement, visible on both CT and MRI (Figure 5A), caused by ‘leaky’ neovascularization and inflammation at the base of the brain; 2) Hydrocephalus (Figure 5A), secondary to CSF resorption obstruction due to inflammatory exudates; 3) Infarctions, supratentorial and in the brain stem (Figure 5A), secondary to oblitative vasculitis (Bernaerts et al., 2003). Examination of cerebral spinal fluid (CSF) is important for the diagnosis TBM as well, but differentiation from bacterial or viral meningitis is often challenging. Suggestive for TBM are low levels of lymphocytes, an increased protein level and a reduced CSF/blood glucose ratio (van Well et al., 2009). For a certain diagnosis of TBM, bacteriological confirmation in CSF is required. However, the paucibacillary nature of TBM results in a low sensitivity of CSF microscopy for acid-fast bacilli (<20%) and CSF culture (<50%) (Solomons et al., 2015). The WHO argues in favor of the use of Xpert MTB/Rif as TBM diagnostic, it is however not favorable to use a single PCR based method as a strategy.
Figure 5. CT and MRI of TBM patients

[A] Transverse section of CT scan with various pathological hallmarks seen in TBM patients: basal meningeal enhancement (arrows), area with infarction (*) and enlarged ventricles representing hydrocephalus. [B] CT scan of TBM patient showing a tuberculoma in the cerebellum (arrow). [C, D] Sagittal (C) and transverse (D) sections of T1 weighted MRI, post gadolinium of 4 year, 11 month old TBM patient with large granuloma in cerebrum. Blood vessels and pathology with high vascularization appear bright on T1 weighted post gadolinium images. Pathological processes with increased fluid collection and edema appear dark. [E, F] Coronal (E) and transverse (F) sections of T2 weighted MRI of the same patient as C, D. Pathological processes with increased fluid collections and edema, as well as cerebrospinal fluid, appear bright on T2 weighted MRI.

General Introduction
In response to the WHO guidelines, a team of experts has come to the conclusion that Xpert MTB/Rif with large CSF volumes in combination with diagnostic tests, clinical findings and if possible radiology is needed to reach the diagnosis as fast as possible and to exclude alternative diagnoses (Bahr et al., 2016; Rohlwink et al., 2016).

Preventive strategies
Prevention of TBM is as important as early diagnosis. Almost a century ago, the attenuated *Mycobacterium bovis* Bacillus Calmette–Guérin (BCG) was developed as vaccine against tuberculosis. This vaccine strain has a partial deletion of the *esx-1* locus. BCG vaccination has shown to be 73% efficient in preventing childhood TBM and miliary TB (Trunz et al., 2006; Wilkinson et al., 2017), but is unfortunately not successful in preventing pulmonary TB and other forms of mycobacterial disease during adulthood. Over the last decades, several preclinical studies showed promising improved vaccine candidates, but none turned out to be successful enough at different stages of clinical trials (Kaufmann et al., 2014). Additional preventive measures should involve the reduction of the many risk factors associated with TB and TBM, like poverty, crowded living conditions, smoking, hypovitaminosis D and HIV co-infection. Several studies report that at least half of the children with TBM were exposed to an adult with proven pulmonary TB in the same household (van Well et al., 2009). Once children are exposed, childhood tuberculosis can be reduced by administering preventive therapy with isoniazid for 6-9 months (Kasaie et al., 2014; Van Soelen et al., 2013). This preventive therapy offers good protection, but therapy compliance is low due to parents who are discouraged to give therapy to a child without any signs of disease (Marais and Schaaf, 2014). Furthermore, hypovitaminosis D is a widespread disorder in developing countries and low levels of vitamin D are suggested to be a risk factor for the development of TB and TBM (Arabi et al., 2010). The association found between sunshine hours and TBM incidence rate in South African children supports this hypothesis (Visser et al., 2012). Finally, HIV co-infection increases the risk on developing TBM and advanced HIV infection leads to a higher morbidity and mortality (Wilkinson et al., 2017). Therefore, the most effective preventive strategy against tuberculosis in HIV co-infected individuals is antiretroviral therapy.

Treatment of TBM
TBM treatment of children is currently based on observational studies and clinical practice instead of large controlled trials, however a consensus has formed to establish a gold standard for treatment. First of all it is of utmost importance to start as early as possible. Treatment should include isoniazid and rifampicin and can have potentially fatal consequences when interrupted during the first 2 months (Thwaites et al., 2013). To prevent relapse, there is a need for long-term treatment, but the optimum treatment length is still uncertain. The general recommendation is a duration of 9-12 months, but
evidence suggests that intensified therapy in HIV-uninfected patients infected with drug-susceptible TB can be safely and effectively administered for 6 months (Heemskerk et al., 2016; Jullien et al., 2016; Mai and Thwaites, 2016). An extra challenge is the alarming increase in patients infected with multi drug resistant (MDR) and extensively drug resistant (XDR) tuberculosis strains. These strains are resistant to first-line drugs or resistant to first- and second line drugs respectively (World Health Organisation, 2010).

For the past decade, immunomodulatory therapy as an addition to standard antibiotics, has gained interest. Corticosteroids have proven to reduce short-term mortality and are recommended as part of the standard regimen (Donald et al., 2016; Prasad et al., 2016). The use of thalidomide, a TNFα-inhibitor, can also lead to clinical improvement and regression of pathology (Tsenova et al., 1998; van Toorn et al., 2015). The effect of immunomodulatory therapy can be explained by its reducing effect on the damaging inflammatory response seen in TBM. However, it has become clear that this therapy is not beneficial for each individual patient due to variation in host genetic and immunological profiles (Thwaites et al., 2013; Tobin et al., 2012).

Outline of this thesis

The aim of the work presented in this thesis is to identify factors that play a role in the early pathogenesis of TBM. For this, I am using both the zebrafish infection model and human neuropathology.

A model that has proven to be a successful addition to study mycobacterial pathogenesis is the Danio rerio – Mycobacterium marinum infection model. Zebrafish develop granulomas upon infection with their natural pathogen M. marinum and these granulomas are highly similar to the pathology found in human tuberculosis, including hypoxia and granulomas with necrotic centers. Furthermore, the translucent zebrafish larvae show great opportunities for real-time imaging. Chapter 2 discusses the unique features of this model and its contribution to TB research.

Chapter 3 describes the in-depth analysis of a M. marinum mannosyltransferase (manT) mutant in the context of innate immunity. This mutant is defective in decorating the cell wall glycolipids lipomannan (LM) and lipoarabinomannan (LAM) with α(1→2) linked mannose residue(s) and is therefore hypothesized to be attenuated in in vitro and in vivo infection models. This mutant was identified during a medium throughput screen in order to identify mycobacterial genes involved in granuloma formation. We show that manT-mutant bacteria were able to complete the macrophage infection cycle similar to wildtype bacteria but nevertheless strongly attenuated after systemic infection of zebrafish embryos. Interestingly, attenuation was less prominent in the context of an adaptive immune system in adult zebrafish.

In Chapter 4 we describe how the zebrafish-infection model is adapted to address the question how this model can be used to study host and pathogen factors involved
in pathogenesis of early CNS granuloma formation. We show that different inoculation routes result in formation of early granulomas in brain parenchyma and meninges in zebrafish larvae in 70-100% of the cases. In addition, 20% of adult zebrafish develop mature granulomas in meninges, representing the typical Rich focus. Infection with an ESX-1 secretion-deficient \( M. \text{marinum} \) strain, resulted in an attenuated phenotype, but these bacteria were still able to cross the blood-brain barrier and infect brain tissue.

Chapter 5 is a follow-up study of chapter 4 and focuses on the question how \( M. \text{marinum} \) cross the zebrafish BBB to establish infection in brain tissue. We hypothesized that mycobacteria can use more than one route to traverse into the CNS. We show that the Trojan Horse mechanism, in which mycobacteria use a phagocyte as carrier, is used as the predominant migration route of \( M. \text{marinum} \) to invade brain tissue. In addition, we propose a novel type of transcellular migration, whereby virulent \( M. \text{marinum} \) infect and damage endothelial cells of blood vessels in an active, ESX-1 dependent manner.

In Chapter 6 we develop and utilize fluorescent tools to study the development of the BBB and blood-choroid plexus (CP) barrier in a living larvae. In order to study this, a reporter line using claudin 5a and GFP was generated with BAC recombineering. With this new transgenic zebrafish line we could show that claudin 5a expressing cells of the CP are ciliated ependymal cell, CP development precedes BBB development and that claudin 5a expression occurs simultaneously with angiogenesis.

The addendum of chapter 6 we hypothesize that CNS invasion by \( M. \text{marinum} \) can occur through both the BBB and blood-CP barrier and that claudin5a integrity is detrimental for successful traversal. To study this, we use a proof-of-principal study to examine the role of Claudin5a in mycobacterial invasion of the CNS. Here we show that systemic infection of zebrafish larvae with an intact BBB resulted in mycobacterial CNS invasion and that formation of early granulomas occurred predominantly near vessels lacking claudin 5a. Furthermore, both systemic and ventricular infection resulted of a large proportion of early granulomas to be formed in the CP.

Chapter 7 describes the in-depth analysis of the ESX-1 secretion system by describing the functional characterization of the accessory ESX-1 proteins EccA\(_1\), EspG\(_1\), and EspH. The individual ESX-1 components were hypothesized to each have distinctive effects on secretion of different ESX-1 substrates, with a subsequent specific effect on virulence. By using secretion analysis, \textit{in vitro} cell infection and \textit{in vivo} zebrafish infections studies, we show indeed that different sets of ESX-1 substrates play different roles at various steps of the mycobacterial infection cycle.

In Chapter 8 and 9 we use a historical cohort of neuropathology and blood samples of TBM patients to study bacterial and host genetics involved in susceptibility to TBM. An analysis of SNPs present in human DNA and its relation with susceptibility and disease progression of TBM is presented in Chapter 8. Here we show a correlation between specific SNPs found in VEGF and Vitamin D related genes and a poor outcome of TBM.
In addition, clinical risk factors for a poor TBM outcome are analyzed in this cohort. Next, we hypothesize that specific bacterial strains from the *M. tuberculosis* complex can be linked to development of TBM. The analysis of bacterial strains involved in TBM is described in chapter 9. We show that lineage 4 is the predominant lineage present in our study population and that infection with more than one lineage occurs in TBM, even in young children.

The results of the studies in this thesis are discussed in chapter 10.


