Summarizing Discussion
Tuberculous meningitis (TBM) is the most severe form of tuberculosis, with an estimated 100,000 cases per year. Despite extensive efforts to improve diagnosis and treatment, management of TBM continues to be suboptimal. For example, diagnosis is often delayed due to the insidious onset of TBM and delay in culture confirmation (Wilkinson et al., 2017). In addition, multi- or extensively-drug resistant strains in combination with poor blood-brain barrier penetration of tuberculostatics impede treatment and compound the burden on public health. Understanding the pathophysiology of mycobacterial CNS invasion and granuloma formation in the CNS is essential to the improvement of TBM management. In this thesis, we present our contribution to identifying early steps in TBM pathogenesis using the zebrafish-\textit{Mycobacterium marinum} infection model and a retrospective cohort of samples from South African TBM patients. Host and bacterial factors contributing to CNS invasion, granuloma formation and risk factors for TBM susceptibility and outcome are investigated and further discussed in the following topics: (1) CNS invasion; (2) Granuloma characteristics in the CNS; (3) Balanced immune response in CNS granulomas linked to TBM and outcome; (4) Translational implications and (5) Future perspectives (Figure 1).

**The zebrafish infection model for tuberculosis and tuberculous meningitis research**

Over the past decade, the \textit{Danio rerio} – \textit{M. marinum} infection model has become a well-accepted and useful model for the study of mycobacterial pathogenesis, as outlined in chapter 2. The transparency of the zebrafish larvae in combination with the (increasing) availability of transgenic zebrafish lines which express fluorescent markers in various cell types makes this an ideal vertebrate animal model for real-time imaging of host-pathogen interactions. Furthermore, \textit{M. marinum}, a close genetic relative to \textit{M. tuberculosis}, causes granulomatous disease in zebrafish with shared characteristics to granulomas in humans (Tobin and Ramakrishnan, 2008). Similarities include the involvement of macrophages which undergo an epithelioid transformation, the formation of a caseous/necrotic core and the importance of specific bacterial virulence factors, like ESX-1 (Tobin and Ramakrishnan, 2008). The use of the zebrafish model has led to important novel insights that contributed to the field of tuberculosis research, which include: 1) Insights into the dynamics of granulomas that aid both host protection and bacterial proliferation (Lesley and Ramakrishnan, 2008; Ramakrishnan, 2013, 2012); 2) the importance of host susceptibility and balanced inflammation for the outcome of disease (Tobin et al., 2012, 2010); 3) newly identified mycobacterial virulence factors and anti-mycobacterial compounds using medium and high throughput screening methods (Makarov et al., 2014; Stoop et al., 2011); and chapter 4) ESX-1 mediated phagosomal escape as an important step in the macrophage infection cycle (Gao et al., 2004; Stamm et al., 2003).
In chapter 4, we demonstrate the capability of the *D. rerio* – *M. marinum* model as suitable to study TBM pathogenesis, thereby answering questions detailing mycobacterial CNS invasion and CNS granuloma composition. We found that 100% of larvae develop early granulomas in the CNS upon local infection (hindbrain ventricle and directly into brain parenchyma) and after systemic infection with *M. marinum*, 70% of cases resulted...
in the development of early granulomas in the CNS. Early granulomas typically locate in relatively close proximity to blood vessels after infection via the blood circulation. Moreover, granuloma formation also occurs in the CNS of adult zebrafish, with granuloma formation in the meninges in 20% of the cases after intraperitoneal infection. Due to the localization of granulomas in close proximity of the meninges, they are likely to be able to induce meningitis and are therefore considered to represent the typical Rich focus, as described in humans (Donald et al., 2005; Rich and McCordock, 1933).

1. Mycobacterial invasion of the central nervous system

Dissemination of M. tuberculosis from lungs to CNS

Mycobacterial involvement of the CNS is a consequence of dissemination of M. tuberculosis from its primary pulmonary focus via lymph nodes or circulation (Pagán et al., 2015; Wilkinson et al., 2017). Why this occurs in specific groups and not in others and how mycobacteria can invade brain parenchyma still remains largely unknown. Nevertheless, dissemination is thought to occur early after primary infection (1-3 months), before adaptive immunity is activated (Thwaites et al., 2013), but the rate of progression is highly variable (Marais and Schaaf, 2014). Occult dissemination is common and usually does not progress to extra-pulmonary disease. However, young children (<3yrs) present with a high risk of disease progression due to their immature immune system relative to adults (Marais and Schaaf, 2014). The early dissemination is likely the reason that children with a BCG vaccination-induced memory T cell response have greater protective immunity against disseminated forms of TB than adults. Similarly, the impaired T cell response seen during HIV infection might explain the increased probability for haematogenous dissemination in this patient group (Thwaites et al., 2013). Pioneering studies by Rich and McCordock have highlighted that a single meningeal or parameningeal granuloma was regularly found to be the entry point for bacteria into the CSF leading to meningitis. Thus providing evidence that TBM is not a direct consequence of miliary TB, which suggests that primary seeding of the CNS by M. tuberculosis is a subclinical event (Rich and McCordock, 1933). However, little knowledge exists surrounding the pathophysiological steps between the primary pulmonary focus and further dissemination in the brain parenchyma. This is especially lacking with regard to the mechanisms utilised by M. tuberculosis to cross the blood-brain barrier (BBB) and enter the CNS.

Mycobacteria employ two different mechanisms to cross the blood-brain barrier

The small group of bacterial pathogens causing meningitis can be subdivided in extracellular pathogens, like Streptococcus pneumoniae, Neisseria meningitides and Haemophilus influenza, and pathogens that predominantly reside intracellular, i.e. Listeria monocytogenes and M. tuberculosis. In general, extracellular pathogens are thought to disseminate directly and induce a substantial inflammatory response with breakdown of
the BBB (Panackal et al., 2016; van Sorge and Doran, 2013). In contrast, the sequence of events in TBM and initial seeding of the brain is most likely non-inflammatory and thus the BBB is thought to be intact (Donald et al., 2016, 2005; Panackal et al., 2016).

With the help of the zebrafish model, that mycobacteria are capable of using two different routes of migration into the CNS (Chapter 5; Figure 2). Thus far our data supports the long-standing Trojan Horse theory in which mycobacteria employ phagocytic cells to cross the BBB. Systemic infection of \textit{M. marinum} in zebrafish larvae with a functionally intact BBB results in rapid phagocytosis of mycobacteria and traversal of infected phagocytic cells across the BBB. During this sequence of events, migration is not characterized by substantial damage to the BBB. Additional evidence for a Trojan Horse mechanism is the observed trafficking of \textit{esx-1} deficient bacteria into the CNS in our model system. \textit{Esx-1} mutants of \textit{M. marinum} were found to traverse to the brain parenchyma after systemic infection, which were always associated with phagocytic cells. Since these mutant bacteria are deficient in completing the macrophage infection cycle, these bacteria are almost exclusively found in the intracellular milieu (Houben et al., 2012; Simeone et al., 2012; van der Wel et al., 2007). \textit{Mycobacterium bovis} BCG, which is also deficient for ESX-1 secretion, is able to invade the CNS in immunocompetent individuals in sporadic cases (Golub et al., 2011). Taken together, this supports the theory of an intracellular (i.e. in macrophages) crossing mechanism in both zebrafish and humans. Moreover, it confirms the first neuropathology findings by Rich and McCordock (Rich and Thomas, 1946; Rich and McCordock, 1933) and subsequent \textit{in vitro} work (Bermudez et al., 2002) that suggested the Trojan Horse mechanism as a first choice crossing strategy.

The Trojan Horse mechanism, in which \textit{M. tuberculosis} uses the macrophage as protective niche, should not trigger a massive inflammatory response. However, a sporadic bacteremia with non-inflammatory deposition of free mycobacteria on the endothelium and invasion of these cells should also not induce substantial inflammation. Observations in chapter 5 show localization of mycobacteria to the endothelium as early as 1 day post infection, supporting the idea that free mycobacteria might be able to attach to the endothelium and cross the BBB in a macrophage independent way. Additional data in chapter 5 supports this theory by demonstrating that \textit{M. marinum} is indeed able to invade endothelial cells without the help of phagocytes, with subsequent damage to the basal lamina and invasion of brain parenchyma. Furthermore, \textit{in vitro} and \textit{in vivo} studies revealed that invasion and infection of endothelial cells by \textit{M. marinum} is an active process dependent on ESX-1 secretion. The capability of \textit{M. tuberculosis} to invade other host cells for early dissemination than phagocytes alone has been described previously. \textit{In vitro} studies suggested lymphatic endothelial cells (Lerner et al., 2016) as a replicative niche for \textit{M. tuberculosis}, and showed the presence of tubercle bacilli in these cells in patient material. Another study described the replication of \textit{M. tuberculosis} in epithelial cells (Menozzi et al., 2006). Most likely, the different migration mechanisms can co-exist;
Figure 2. Potential mechanisms involved in mycobacterial CNS invasion.

[A] Schematic summary of the findings in chapter 5, illustrating the two mechanisms employed by mycobacteria to invade the CNS: (1) phagocyte dependent, in which mycobacteria use a phagocyte as carrier to traverse an intact BBB, and (2) phagocyte independent traversal where mycobacteria invade and subsequent damage endothelial cells in an active ESX-1 dependent way. [B] Schematic representation of the hypothetical crossing mechanisms employed by mycobacteria to cross the endothelial and ependymal barriers in the blood-CSF barrier. In theory, they might use a macrophage dependent mechanism, similar to the Trojan Horse mechanism to cross the BBB or an alternative crossing mechanism, which can be transcellular or paracellular.
meaning that transcellular crossing of free mycobacteria via endothelia and the Trojan Horse mechanism can initiate mycobacterial migration across the BBB simultaneously.

The role of the blood-brain barrier and vascular specificity in mycobacterial CNS invasion

The blood-brain barrier is a unique host factor to the CNS, thus far little is known about its exact role during mycobacterial trafficking. We have shown that the BBB restricts but not completely blocks mycobacterial migration into the brain followed by the observation that early CNS granulomas are formed even when a functionally intact BBB is present (Chapter 4 & 5). In chapter 6 we describe a new transgenic zebrafish line that provides a valuable new tool to study the specific role of the BBB in mycobacterial pathogenesis. We show that the gene claudin 5a is the zebrafish orthologue of human claudin5. Expression of claudin 5a is found in brain vasculature defining the BBB and in ciliated ependymal cells of the choroid plexus (CP). Using our transgenic zebrafish line, we could show that CP development starts as early as 1 day post infection (dpf) in zebrafish larvae and precedes BBB development, which starts at 2 dpf, and that stable GFP expression is maintained in brain tissue of adult zebrafish. Furthermore, we could validate our model by showing that claudin 5a expression occurs simultaneously with angiogenesis with the use of in vivo imaging, further corroborating previous findings (Umans et al., 2017). Furthermore, the cells expressing claudin 5a drive the fluid flow in the brain ventricles. In the addendum of chapter 6, we performed a proof-of-principle study to demonstrate the usage of this line in studying the role of the BBB in mycobacterial CNS invasion. Interestingly, systemic infection resulted in mycobacterial CNS invasion and formation of early granulomas predominantly near vessels lacking claudin 5a expression. This suggests that infected macrophages have a preference for traversing endothelial cell layers that are not reinforced by Claudin 5 tight junction, possibly at a spot in the vessel wall with least resistance. The CNS vasculature is highly heterogenic and vessel specific roles in physiology and pathophysiology have been suggested before (Wilhelm et al., 2016). Brain microvascular endothelial cells (BMECs) of venules are thought to be involved in inflammatory-related processes, based on their expression profile in vitro (Macdonald et al., 2010). Additionally, these cells were suggested to have a looser organization of tight junctions, compared to capillaries (Macdonald et al., 2010), which seems to indicate that (infected) macrophages encounter less resistance when leaving the blood stream at these specific spots. This is of particular interest, as we were able to show a preferred crossing of bacteria near veins rather than arteries. However, a clear distinction between arteries, veins and capillaries is challenging in developing zebrafish larvae and would need the specific labeling of the different BMEC types. Furthermore, mycobacterial invasion of the CNS was observed predominantly at spots without claudin 5a labeling, which suggests an absence instead of loose organization of tight junctions. However, it should
be noted that Claudin 5a is the only tight junction that is visualized in this transgenic line and that we did not use counterstains for other tight junction proteins, like ZO-1 (Fleming et al., 2013). Therefore, we cannot exclude the possibility of other tight junctions present and functional at spots lacking Claudin 5a labeling.

**Does the blood-CSF barrier in the choroid plexus serve as alternative passageway into the CNS?**

Previous studies have solely focused on the BBB, yet the role of the blood-CSF barrier is as of yet largely understudied. This barrier, formed by the epithelial cell layer of the CP between blood and ventricular CSF, is also highly interesting in the context of infectious diseases involving the CNS. The CP harbors various immune cells, including CP macrophages and dendritic cells (Lun et al., 2015) and is considered to provide immune cell passage across the blood-CSF barrier into the CNS, particularly early in life (Lun et al., 2015; Saunders et al., 2012; Shechter et al., 2013). In some cases, ependymal granulomas have been found in human autopsy material and experimental animal models (Rich and McCordock, 1933; Rock et al., 2008). This raises the question, whether mycobacteria might be able to use even a third CNS invasion route, namely the CP. After CNS entry via the CP, free or phagocytosed bacteria might spread across the brain surface via the ventricular CSF flow, which originate in the CP, to establish granulomas in the meninges. The opposite can also be true, because CSF movement cannot be considered as a strong one directional flow. Several experimental models showed that particles injected directly into the CSF can find their way back to the ventricles and CPs (Turner et al., 2012), meaning that CP granulomas can also be formed by mycobacteria originated from meningeal granulomas.

Support for the involvement of the blood-CSF barrier in the CP in mycobacterial CNS disease can be found in chapter 4 and the addendum of chapter 6 of this thesis. In chapter 4, local inoculation of *M. marinum* in the hindbrain ventricle resulted in the formation of infectious foci in the entire ventricular system and brain parenchyma in all larvae. This indicates that bacteria are able to cross from CSF to brain tissue, presumably via the choroid plexus or directly through the meningeal layer. However, these larvae were infected at 2 days post infection, before the presence of a functionally intact barrier. In the addendum of chapter 6, the same experiment was performed at 4 dpf, *i.e.* after formation of the BBB and blood-CP barrier. This resembles a situation in which the CNS barriers are considered to be functionally intact (chapter 4, Xie et al., 2010; Fleming et al., 2013). Infection at 4 dpf resulted in single bacteria and early granulomas in the diencephalic CP (dCP) and myelencephalic CP (mCP). More intriguingly, single bacteria were found to co-localize with ependymal cells and **claudin 5a** expression in tight junctions. In addition, systemic infection resulted in formation of many granulomas in or near the CP, suggesting that single bacteria and infected macrophages can easily cross
the blood-CSF barrier in both directions. It is not clear from our data which signals are involved in cell recruitment and why the CP is a preferred place for mycobacteria to reside, yet the observations that many immune cells reside within the CP might explain this phenomenon in part. In addition, more experiments are necessary to show which exact migration mechanism is used by mycobacteria to cross this barrier, but based on our preliminary data, we speculate that all three described mechanisms, Trojan Horse, paracellular and intracellular, are possible (Figure 2, (Kim, 2008)).

*M. tuberculosis* virulence factors are important for BBB crossing

In chapter 4 and 5, we reveal important fundamental aspects of CNS invasion in which bacterial virulence factors are clearly essential determinants. Infection with a *M. marinum* mutant deficient for ESX-1 secretion was used to study the effect of this well-known virulence factor in the zebrafish infection model for TBM and to study its potential effect on CNS invasion. First, we show that our ESX-1 mutant is severely attenuated in the CNS, through reduced granuloma formation and scattered isolated phagocytic cells. These phagocytic cells in the CNS of larvae have a high bacterial load, which is in line with previous studies (Davis and Ramakrishnan, 2009; Stoop et al., 2011; Volkman et al., 2004). Next we reveal that invasion and infection of endothelial cells by *M. marinum* is an active process dependent on ESX-1 secretion, extending its function beyond the role of phagosomal escape.

Considerable genetic variation in infecting *M. tuberculosis* strains in TBM patients has been described previously (Caws et al., 2008; Faksri et al., 2011; Pan et al., 2015; Ruesen et al., 2018). In chapter 9, we used a retrospective patient cohort to show an overrepresentation of lineage 4 in CNS samples of TBM patients and demonstrated the presence of more than one lineage (genotypic heterogeneity) in 20% of the patients. Interestingly, previous work suggested that strains from lineage 4 are less likely to cause extra-pulmonary disease and might even be relatively protective (Caws et al., 2008; Faksri et al., 2011). Partially because of this, most research focused on a possible link between Beijing isolates, belonging to lineage 2, and TBM pathophysiology. For example, a recent *in vitro* study demonstrated bacterial dissemination through secretion of vascular endothelial growth factor (VEGF) during macrophage infection with *M. tuberculosis* (Polena et al., 2016). Moreover, infection with the highly virulent Beijing isolate induced higher VEGF levels, where infection with the ESX-1-deficient *M. bovis* BCG strain resulted in reduced VEGF production. Increased VEGF levels were linked to a higher rate of bacterial dissemination and extra-pulmonary manifestation of disease by the Beijing isolate (Polena et al., 2016).

In order to find bacterial genes involved in TBM pathogenesis and explain the fundamental pathophysiological mechanisms, a better approach might be to focus on variation of individual genes instead of variation between the seven large sequence
polymorphism--based lineages. An example of genetic variation not restricted to a lineage is a single nucleotide polymorphism (SNP) in the PE_PGRS33 gene. PE_PGRS33 is a secreted and cell surface localized protein that has been implicated in macrophage entry of *M. tuberculosis* via interaction with TLR2 (Palucci et al., 2016). *In vitro* and *in vivo* experiments supported the fundamental role in CNS invasion, after this SNP was found to be of clinical importance in development of TBM in Chinese children (Wang et al., 2011). Several additional mycobacterial genes linked to CNS specific phenotypes were identified in *in vitro* and *in vivo* studies (Be et al., 2012, 2008; Jain et al., 2006; Ruesen et al., 2018), however the clinical implication of these genes has as of yet not been confirmed. Of particular interest is *Rv0931c*, encoding for the serine/threonine protein kinase PknD. Studies in mice and guinea pigs revealed its importance for invasion of brain endothelial cells specifically, presumably via interaction, likely through phosphorylation, with CNS-associated laminin isoforms (Be et al., 2012). This effect was neutralized by specific antisera and the use of a vaccination, suggesting its potential function in preventing dissemination to the CNS (Skerry et al., 2013) (Be et al., 2012). Unfortunately, a *pknD* mutation has not been described in clinical isolates at the time of writing. Another gene, *Rv0218*, was recently suggested to be of importance in CNS invasion because a higher prevalence of bacterial strains with mutations in this gene was found in TBM patients (Ruesen et al., 2018). In theory, the *Rv0218* mutation alters the surface of *M. tuberculosis* providing it with mechanisms to evade the host immune response and allow for extrapulmonary dissemination. However, in order to use interesting candidates like these to develop novel therapeutic approaches, more *in vivo* fundamental studies are necessary.

2. **Host and bacterial factors involved in granuloma formation**

*Essential role for microglia in the formation of early CNS granuloma*

Once mycobacteria reach the CNS they induce the formation of granulomas, the pathological hallmark of TB. In chapter 4, we studied the characteristics of early granulomas in the CNS of zebrafish. Clusters were found in parenchyma or meninges and were composed of a uniform population of both epithelioid and foamy macrophages, and some heterophiles (the zebrafish neutrophil equivalent). No necrosis or fibrosis was found, alluding to early granuloma formation where no maturation had taken place. Although we know that the main mammalian cell types are highly conserved in zebrafish ((Oosterhof et al., 2015; Renshaw and Trede, 2012), chapter 2), the number of available specific antibodies to distinguish these cells are limited. Several studies, including chapter 4 of this thesis, speculate about the pivotal role of microglia in regulating the immune response within the CNS (Spanos et al., 2015). Microglia are the resident immune cells of the CNS, separated from the circulation by the blood-brain barrier and are thought to be the first line of defense against injury and infection of the CNS (Oosterhof et al., 2015; Spanos et al., 2015). In early larval development the primitive yolk sac-derived macro-
phages expressing the common leukocyte marker L-plastin, colonize brain and retina. Between 2 and 3 dpf a phenotypic transition occurs with a subsequent down-regulation of L-plastin expression and increased expression of apolipoprotein-E (Herbomel et al., 2001, 1999; Meijer and Spaink, 2011; Oosterhof et al., 2015). *In vitro* studies support the theory that microglia play an important role in defense against *M. tuberculosis*, by showing bacterial uptake in 15% of astrocytes and in up to 76% of microglia (Peterson et al., 1995; Rock et al., 2008; Spanos et al., 2015). Although other cell types can be infected, examples include astrocytes and neurons, infection efficiency is much lower in these cells. In chapter 3 we show a clear decreased intensity in L-plastin signal in a proportion of cells in early CNS granulomas, but a specific microglia marker was not used. Therefore, we can only speculate that these cells represent microglia. To further study the role of specific cell types in early granuloma formation, follow up experiments should include the combination of transgenic zebrafish lines with fluorescently labeled peripheral macrophages (MPEG, (Ellett et al., 2011)), neutrophils (MPX, (Renshaw et al., 2006)) and microglia (APO-E, (Peri and Nüsslein-Volhard, 2008)). This will allow for real-time imaging of the events leading to granuloma formation and will aid in distinguishing cell types involved.

**Development of different types of mature granulomas**

In chapter 2 and 3, we describe the formation of granulomas in adult zebrafish, with a fully mature immune system, in the abdominal organs and the CNS. Granulomas were composed of epithelioid and foamy macrophages with mycobacteria present in macrophages. Moreover, signs of maturation, like the presence of lymphocytes and central necrosis of macrophages, were observed. Extra-pulmonary granulomas tend to exhibit the same architecture as pulmonary granulomas, yet little is known about the composition of CNS granulomas. The CNS has been considered to be immune privileged for long, which might suggest that TB in the brain has different characteristics when compared to infection localized in other areas. However, many recent reports show that the CNS is certainly not immune privileged and contains a variety of immune cells and even a lymphatic system (Kipnis and Filiano, 2017; Oosterhof et al., 2015; Panackal et al., 2016; Wood, 2015). Furthermore, based on our research and early studies by Rich and McCordock, CNS granulomas probably share many characteristics with the general granuloma composition, like foamy macrophages, central necrosis and the presence of lymphocytes at the periphery of granulomas (Cadena et al., 2017; Ramakrishnan, 2012; Rich and McCordock, 1933).

CNS granulomas in patients can be classified in three different types based on their histological and radiographic appearance: non-necrotizing, necrotizing with a solid center (gummatous) or necrotizing with a liquid center (abscess) (Mattila et al., 2013). The question remains if these granuloma types are different end points of the disease or
perhaps represent different stages of the disease. Moreover, growing evidence suggests that granulomas are not static entities, but dynamic structures progressing from one type to another over time. Therefore a balanced immunological response is essential for a favorable outcome of disease. Studies in non-human primates demonstrated that TB pathology can be highly diverse and that sterile granulomas and granulomas with active disease can co-exist even within a single individual (Cadena et al., 2017; Flynn et al., 2011). Interestingly, granulomas in adult zebrafish also defer in form within a single zebrafish or between zebrafish infected with different bacterial strains. Thereby indicating that this phenomenon is both affected by variation within the host as well as by bacterial variance. It is important to understand the dynamics of these different granulomas and the factors that play a role in this process. This will have significant implications for treatment strategies and can explain between-patient variation in outcome. The zebrafish infection model could help to unravel this aspect.

**ESX-1 secretion in TB and TBM pathophysiology**

Besides host factors, specific bacterial factors, like ESX-1 secretion, are important in (the initiation of) granuloma formation. The ESX-1 secretory machinery consists of ESX-conserved components (Ecc) EccB1, EccA1, EccD1, EccE1, and is stabilized by MycP1 protein (Ates et al., 2016; Bitter et al., 2009; Van Winden et al., 2016). The secretion system requires cytosolic components EspG1 and EccA1, which are highly conserved among pathogenic mycobacterial species (Ates et al., 2016). The gene espH, distributed between espG1 and eccA1 is unique to the esx-1 locus, but little is known about its exact function. In chapter 7, we characterize the individual ESX-1 components EccA1, EspG1 and EspH, which were suggested to be of importance in infection of mammalian phagocytic cells (Weerdenburg et al., 2015). These components were identified with the use of transposon-directed insertion site sequencing (TraDIS), a method to test a library of transposon mutants in a single experiment and subsequently quantify all mutants using second-generation sequencing methods (Weerdenburg et al., 2015). This experiment is difficult to perform directly in zebrafish larvae, due to the number of infected zebrafish required to get a good representation of the library, but has been used efficiently to determine virulence factors required for the survival of *M. marinum* in in phagocytic host cells (Weerdenburg et al., 2015). Strikingly, using this analysis method these mutants were attenuated in mammalian cells and showed hypervirulence in two different amebas. Therefore, we decided to produce these mutants and test them in our zebrafish infection model.

We showed that single deletion of the related genes affected the secretion of ESX-1 dependent substrates at different levels. Deletion of *espG1* in *M. marinum* results in a complete loss of secretion of all known ESX-1 dependent substrates, whereas deletion of *eccA1* only led to minor secretion defects, which was also dependent on its growth
medium. Finally, \(espH\) deletion resulted in a complete blockage of EspE and EspF secretion and also affected EsxA/EsxB secretion. These secretion defects were linked to different virulence patterns in \textit{in vitro} cell infection and \textit{in vivo} zebrafish infection experiments: EspG\(_1\) and EspH, but not EccA\(_1\), play a major role in early stages of infection \textit{in vitro} and \textit{in vivo}. Surprisingly, EspH seems to have a host specific or \textit{in vivo} specific effect, illustrated by a hypervirulent phenotype in zebrafish larvae but not in cell infections \textit{in vitro}. Therefore our data indicate that EspH is not required for initial phagocytosis, recruitment of cells and primary establishment of early granulomas, but EspH seems essential for the maintenance of a stable granuloma. Because the \(espH\) mutant is defective in a subset of ESX-1 substrates, these experiments also underscore that ESX-1 substrates have different functions in the infection process (chapter 7).

Testing these mutants in the zebrafish model for TBM, to study whether these effects are similar in the CNS and if these factors are involved in initial CNS invasion would provide essential additive information to this study. Hypothetically, it seems likely that the \(espG_1\) mutant, like the eccCb\(_1\) mutated strain used in Chapter 4 and 5, is essential for invasion of endothelial cells. In addition, the eccA\(_1\) mutant probably resembles a wild-type (WT) infection, meaning that this gene is probably not essential for BMEC invasion. Deletion of \(espH\) resembles infection with the \(espG_1\) mutant strain \textit{in vitro}, which alludes to the fact that EspH is essential for invasion of BMECs in a macrophage-independent way. However, once macrophages establish a granuloma, the \(espH\)-deficient strain seems to be hypervirulent, illustrated by increased cell death and extra-cellular bacterial growth. Thereby suggesting that EspH plays a role in granuloma establishment and maintenance and possibly macrophage recruitment. The function of EspE/EspF, the two proteins that are most severely affected by the \(espH\) deletion is as of yet not studied in \textit{M. tuberculosis}. However our results shed light on the potential importance of EspH with regard to interaction with the host to induce a homeostatic balance between in developing granulomas. Our observations add to the long-standing idea that ESX-1 secretion in general is essential for dissemination, granuloma formation and macrophage recruitment. Moreover, it has the potential to specify and attribute a specific part of mycobacterial pathogenesis to an individual ESX-1 component.

\textit{Additional bacterial virulence factors involved in early granuloma formation}

The zebrafish larval model is an excellent model to study and rapidly screen for bacterial virulence factors involved in early granuloma formation. The zebrafish model was previously used to screen a transposon mutant library of \textit{M. marinum} which identified mycobacterial genes involved in the onset of granuloma formation (Stoop et al., 2011). In chapter 3, we further characterized the role of one of the identified mutants. We analyzed an \textit{M. marinum} mutant that is unable to produce mannosyltransferase (\textit{manT}), an enzyme involved in the biosynthesis of lipomannan (LM) and lipoarabinomannan (LAM),
which are glycolipid constituents of the mycobacterial cell wall. Our data revealed an attenuated phenotype in embryos infected with the \textit{manT} mutant compared to wildtype infection. Injection of bacteria into the hindbrain ventricle, a relatively immune privileged site, showed that this phenotype was not a consequence of reduced recruitment of macrophages to site of infection. In addition, \textit{manT} mutant bacteria were able to complete the macrophage infection cycle similar to wildtype bacteria. Interestingly, attenuation was less clear in adult fish, thus providing an insight into the adaptive immune system as opposed to the innate response highlighted by larvae. Since several studies suggest an interaction between mannan branching and innate TLR-2 activity (Nigou et al., 2008), the observed phenotype might be explained by an effect in the context of innate and not adaptive immunity. This highlights again that the unique possibility to study mycobacterial infection solely in the context of innate immunity contributes to our understanding of these factors in virulence.

3. Susceptibility and outcome of TBM

The necessity of a balanced inflammatory response

Increasing evidence show that a balanced inflammatory response upon infection is essential for TBM susceptibility and outcome. This hypothesis is supported by observations of increased susceptibility in immunocompromised conditions like HIV-co infection, deficiency of Tumor necrosis factor α (TNFα), Interferon-γ (IFNγ) or Interleukin-12 (IL-12) (Dissanayeke et al., 2009; Ramakrishnan, 2012; Tobin et al., 2012, 2010; Wilkinson et al., 2017). These conditions result in an inadequate response upon infection with reduced inflammation, extensive bacterial growth and disorganized granulomas. The other end of the spectrum is a pro-inflammatory response resulting in a hyperinflammatory state, leading to collateral damage of local tissue (Pagan and Ramakrishnan, 2015; Tobin et al., 2010). Several host genetic polymorphisms affecting the innate immune response and immunological balance are linked to extra-pulmonary manifestation of TB and severity of TBM (Caws et al., 2008; Dissanayeke et al., 2009; Hawn et al., 2006; Thuong et al., 2007; Wilkinson et al., 2017). An interesting example of a gene locus affecting TNFα levels with subsequent effect on the inflammatory balance within a granuloma is \textit{LTA4H} (Flynn et al., 2011; Pagan and Ramakrishnan, 2015; Tobin et al., 2010). Studies in zebrafish and TBM patients have identified the role of this locus, which controls the balance of pro- and anti-inflammatory eicosanoids. A dysregulation of this balance results in either inadequate inflammation caused by excess of lipoxins and downstream reduced TNFα or hyperinflammation driven by excess leukotriene B(4) and high levels of TNFα. Surprisingly, homozygosity for this gene locus of both the minor or major allele resulted in increased disease susceptibility. Heterozygosity was suggested to form an advantage by protecting against disease, leading to a higher survival rate compared to homozygosity within a Vietnamese cohort among 182 TBM patients (Tobin et al., 2012). Unlike this study, we
could not identify a significant association between outcome and LTA4H genotype in our retrospective cohort study (chapter 8). This could be due to our small sample size or the effect might be population specific. An Indonesian study with 427 TBM patients also failed to show a relation between LTA4H rs17525495 and disease outcome, meaning that this link is not as straightforward as initially suggested (Van Laarhoven et al., 2017). Additionally, van Laarhoven et al. showed a trend between LTA4H rs17525495 TT genotype and better survival in a subgroup of patients with milder disease. Our study did reveal a trend for LTA4H rs2660898 GG genotype and poor outcome in a patient group where the majority of patients presented with a high TBM stage. Therefore, effects of LTA4H polymorphisms might be dependent on ethnic diversity and disease stage.

Specific genetic polymorphisms affecting interleukin (IL) 4/IL13 signaling are associated with outcome

In chapter 8, we set out to study both clinical and genetic risk factors, additional to the LTA4H locus, associated with TBM outcome. Based on previous reports and results within our study consortium, we selected genes affecting the pro- and anti-inflammatory cytokines TNFα, Interleukin-4 and 13 (Visser et al., 2014), growth factor vascular endothelial growth factor (VEGF) (Datta et al., 2015; Misra et al., 2012; Oehlers et al., 2016, 2014; van der Flier et al., 2004; Visser et al., 2014) and the hormone Vitamin D (Liu et al., 2006; Visser et al., 2014). Within the here described group of South African children and adults, we could identify polymorphisms in genes related to the Vitamin D pathway (VDR rs7975232 and PPP6R3 rs7109294), VEGF signaling (VEGFR1 rs9554316 and VEGFA rs833061) and IL4/IL13 signaling (IL13 rs2066960, IL4R rs1805015), to be more prevalent among patients with a poor outcome. In addition, convulsions, focal neurological signs, high TBM stage at presentation, low CSF cell counts and infarctions were found to be clinical risk factors for poor outcome during a 6 month follow up period.

It should be noted that it is challenging to directly link the observed polymorphisms to a significant biological effect in this small population. Nevertheless, we could show an association between these SNPs and good or poor outcome, which can direct future research to further explore their biological significance. Potentially, all SNPs alter their associated pathway and thereby affect the inflammatory response to infection with M. tuberculosis. For example, SNPs affecting the IL-4/-13 - IL-4Rα pathway can direct the balance between Th1- and Th2-cells to a predominant pro- (Th1) or anti-inflammatory response (Th2). In the context of TB, SNPs in genes encoding these cytokines were linked to reactivation of disease and increased lung damage (Heitmann et al., 2014; Hölscher et al., 2016; Van Crevel et al., 2000). Chapter 8 is the first genetic association study which focuses on SNPs affecting the IL-4/-13 - IL-4Rα pathway in TBM patients, however elevated levels of IL-4 or IL-13 in the CSF have been associated with TBM before, suggesting a potential link. Increased levels of IL-4, among other pro-inflammatory cytokines, were
associated with hydrocephalus and infarction (Sharma et al., 2017). In addition, examination of CSF of our patient group was performed during previous research in our study consortium (Visser et al., 2014), and identified elevated IL-13 levels in the CSF. Increased levels of this anti-inflammatory cytokine was found in TBM patients specifically and not in patients with bacterial or viral meningitis. Hypothetically, SNPs can either increase or decrease IL-13 levels with a subsequent hypo- or respectively hyperinflammatory response. Moreover, SNPs have the potential to alter the balance in a predominantly pro-inflammatory (IL-4) or anti-inflammatory (IL-13) phenotype. Unfortunately, we could not identify an association between IL-4 or IL-13 levels and genotype, due to the small sample size in the study described in this thesis.

SNPs in VEGF related genes hypothetically affect angiogenetic balance and vascular pathology

Additional interesting findings in our study are polymorphisms affecting the VEGF-pathway, which might have effects on the presence of vascular pathology, like angiogenesis, vasculitis and infarction, commonly found in TBM (Donald et al., 2016; Kumar et al., 2016; van der Flier et al., 2004), chapter 8). VEGFA binds VEGFR1 and VEGFR2 which are both highly expressed on endothelial cells and upregulated in response to mycobacterial infection (Kumar et al., 2016). VEGFR2 is known to drive angiogenesis after binding by VEGFA (Datta et al., 2015; Oehlers et al., 2014), which mediates extrapulmonary dissemination of M. tuberculosis (Polena et al., 2016) and might play a role in CNS invasion (chapter 4). VEGFR1 most likely modulates VEGFR2 signaling and restricts the angiogenetic signal, however less is known about the exact function of this receptor (Jeltsch et al., 2013; Kumar et al., 2016). If both VEGFR1 and VEGFR2 are necessary for a balanced angiogenetic response, one might speculate that alterations of these signals result in a dysregulation of homeostasis. Decreased VEGFR1 signal might lead to a reduced restriction of angiogenetic signal and therefore increased neovascularization. The observation that we could link certain polymorphisms in VEGF pathway related genes to TBM outcome might suggest that these polymorphisms indeed affect VEGF balance and therefore TBM immunopathology and outcome, but again no association was identified with VEGF levels in blood or CSF described in previous work in the same patient group (Visser et al., 2014).

The zebrafish model is an excellent tool to perform additional functional studies to research the potential effect of these polymorphisms. In the zebrafish model, the importance of VEGFA in TB pathogenesis and neovascularization has been confirmed and anti-VEGF treatment has been suggested to resolve vascular leakiness and improve survival (Oehlers et al., 2016, 2014). Chapter 4 suggests a potential role for the VEGF pathway in CNS invasion of M. marinum by showing co-localization of intensified kdr1/ vegfr2 signal with M. marinum-loaded phagocytes in larval brain blood vessels. This
might suggest an interaction between infected macrophages and the blood vessel wall mediated by secreted VEGF and/or affecting VEGFR2 activity. However, our manipulation of the VEGF signal in larvae resulted in altered levels of overall infection but did not affect the percentage of infected phagocytes invading the CNS, which seems to indicate that this strategy is not as simple as initially thought. Possibly, other factors are involved like activation of VEGFR1 as alternative route or the TNFα pathway interacting with VEGF, nonetheless additional research is necessary to elucidate this complex interplay of pathways.

4. **Translational implications**

*Novel therapeutic approaches based on individual immunological profile*

With the rising *M. tuberculosis* resistance against antimicrobial drugs, modulation of immune pathways that impact on inflammation and immunopathology have the potential to improve the treatment of TBM. So far, chemotherapeutic agents most commonly used and most accepted are corticosteroids, like dexamethasone and prednisone, which reduce overall inflammation and improve survival rates in HIV-1 negative patients. However, no effect on long-term neurological sequelae was found so far (Donald et al., 2016; Prasad and Singh, 2008) and overall reduction of inflammation is not beneficial in the proportion of patients with a predominantly hypo inflammatory genetic profile. Specific drugs, like thalidomide or infliximab, which reduce TNFα production, may provide an alternative but will again only benefit a specific subset of patients. Moreover, although experimental models show effectiveness of TNFα blocking agents in the treatment of TBM, the application in pediatric TBM has not proven to be successful (Schoeman et al., 2004; Tsenova et al., 2002, 1998). A promising candidate that gained recent attention is anti-VEGF treatment, for example pazopanib (VEGFR inhibitor) or bevacizumab (anti-VEGF antibody). As mentioned before, experimental models suggest that anti-VEGF treatment resolve vascular leakiness, reduce hypoxia and reduce bacterial load in zebrafish (Oehlerls et al., 2016, 2014). In addition, treatment with bevacizumab of rabbits infected with *M. tuberculosis* resulted in vascular normalization in granulomas, thereby improving delivering of antimicrobial drugs (Datta et al., 2015). Although an effect of VEGFR2-blocking or VEGFA-induction on overall infection level was seen in our TBM study in zebrafish larvae (Chapter 5), manipulation of VEGF did not seem to prevent mycobacterial dissemination to the central nervous system. This suggests that manipulation of this signal might only affect later stages of disease, in line with previous results. Additionally, chapter 8 suggests once more that only a subset of individuals might benefit from anti-VEGF therapy. Therefore, identifying patient group with specific genetic profiles is of utmost importance to improve the TBM therapeutic approach.
Immunomodulatory effect of Vitamin D as possible preventive measure

An interesting, inexpensive, host-directed therapy might be Vitamin D supplementation. Hypovitaminosis D is a widespread disorder in developing countries and Vitamin D has been suggested to have immunomodulatory effects in TB pathogenesis (Arabi et al., 2010; Tobin, 2015). Interestingly, chapter 8 suggested polymorphisms affecting the Vitamin D pathway (VDR rs7975232, PPP6R3 rs7109294, CYP2R1 rs10741657 and CYP27B1 rs703842) as most promising candidates for clinical importance in TBM progression and outcome. It should be noted that this association study was performed in a small patient cohort, resulting in a possible over- or underrepresentation of the actual effect in a larger population. However, our results shed light on the potential importance of SNPs affecting Vitamin D signaling in TBM outcome. Vitamin D levels have been proposed to play an important role in the pathophysiology of diseases with long latency, such as TB and TBM (Nnoaham and Clarke, 2008; Wagner et al., 2008). In particular, low vitamin D levels in patients with pulmonary TB have been associated with reactivation of latent disease. In the context of TBM, significant associations between reduced sunshine hours and increased incidence of TBM have been found in a retrospective TBM cohort in South Africa (Visser et al., 2012), suggesting a role for Vitamin D levels. Additionally, elevated levels of cathelicidin LL-37 were found in serum and CSF of TBM patients compared to patients with bacterial or viral meningitis in our patient cohort (ref). This implies monocyte activation by *M. tuberculosis* which induces VDR-dependent production of antimicrobial peptides, such as cathelicidin LL-37 (Liu and Modlin, 2008; Visser et al., 2014). However, prerequisite for activation of the Vitamin D - VDR complex and an adequate immune response are sufficient levels of Vitamin D. Although supplementation of low levels of Vitamin D may provide a promising strategy for preventive actions, clinical trials did not show a clear effect of Vitamin D administration on susceptibility to TB in general thus far (Tobin, 2015). An association between the TaqI VDR polymorphism and sputum conversion in a study population of pulmonary TB patients with high dose antibiotics and vitamin D(3) administration, is the only association found (Martineau et al., 2014), and suggests a potential effect in subgroups.

5. Future perspectives & concluding remarks

The work described in this thesis has provided insight in both host and bacterial factors involved in early steps of CNS TB, but many questions have yet to be resolved. It is for example still largely unknown why certain individuals develop TBM and others develop pulmonary TB alone, the discrepancy between children and adults, the exact role of Vitamin D, VEGF or bacterial strains in TBM pathophysiology and finally how BBB integrity changes during the non-inflammatory and inflammatory stages of TBM. Clearly, CNS immunopathology cannot be explained by host or bacterial factors individually. Our retrospective cohort study hints to specific bacterial and host factors that are involved,
However association studies to detect clear associations should consist of a larger population. Substantially larger TBM studies have been conducted in (South) East Asia but have not been performed in South Africa at the time of writing. Based on the numerous reports about geographical differences in bacterial strain dissemination and genetic polymorphisms, studies in South Africa have the potential to add to the existing data and increase our knowledge about TBM pathophysiology. In addition, HIV co-infection is a factor affecting TBM outcome, which has the highest prevalence in South Africa and should be considered when studying TBM pathophysiology. Furthermore, it is important to consider the interplay between both host and bacterial genetics in the study of TBM susceptibility. Based on this thesis, follow-up studies should focus on the BBB, VEGF and vitamin D and their role in TBM pathophysiology and on how to use this information to improve host-directed therapy.

At first, clinical studies should focus on the role of VEGF and Vitamin D and should be a combination of host and possibly bacterial genetic polymorphisms, pathway related markers in CSF and/or blood, clinical data and radiographic imaging. Sequential MRI/PET-CT imaging of both TBM patients and zebrafish to follow granuloma formation and the development of vascular pathology over time and combine this with analysis of specific (VEGF related) polymorphisms in patients would be of great benefit. Following upon this, the zebrafish model presents a unique tool to study the fundamental basics explaining this pathology and could be used to test potential host-directed therapeutic strategies. In the context of Vitamin D, a prospective study should include the determination of our identified SNPs in combination with Vitamin D and vitamin D related biomarker (like LL-37) levels in blood and CSF in a larger cohort, with the aim to identify patient groups that potentially benefit from Vitamin D supplementation. Since zebrafish possess a vitamin D-VDR pathway similar to human with VDR widely distributed in all tissue from 48 hpf onwards, this model is again an useful tool for experimental studies (Craig et al., 2008).

Second, still little is known about the function of the BBB and blood-CP barrier during initial mycobacterial CNS invasion and manifestation of TBM. This is in particular interesting in the context of the increased risk seen in early life. It has been speculated that the immaturity of the BBB during early childhood contributes to the higher incidence of TBM in young age, but this topic is under debate (Saunders et al., 2012). Although we know little about the BBB developmental stages in early childhood, we do know that in mice the expression of claudin molecules and the functionality of the BBB is reached between day 12 and 15, i.e. well before birth (Nitta et al., 2003; Saunders et al., 2012; Steinemann et al., 2016). Also in zebrafish, a functional BBB is present in early developmental stages (Fleming et al., 2013; Jeong et al., 2008; Xie et al., 2010) and we have indications that BBB integrity is not compromised during early CNS invasion (Chapter 5). Therefore, one might argue that this ‘immaturity’ of the BBB is in...
fact not the reason that young children are at increased risk of developing TBM. For example, there may be a link with specific genetic polymorphisms affecting BBB integrity in specific risk groups, in combination with other risk factors like a relative immature immune system and living conditions. If this is indeed the case, studies to research this should include the determination of polymorphisms in BBB related genes, like claudins or occludins, and functional knockout of specific BBB genes in zebrafish, like claudin 5a or newly identified genes. Increasing our knowledge about these early essential events in pathophysiology can help us to explain variation in pathological and clinical presentation. Moreover, BBB integrity during initial granuloma formation without meningitis and during active TBM is an important topic in the context of (host-directed) therapy. Once promising new treatment strategies are developed that work on pulmonary TB, the question remains if this will be delivered into the CNS when the BBB is still intact. A method used to study bevacizumab (anti-VEGF) drug delivery for the treatment of diffuse intrinsic pontine glioma, a condition in which the BBB is usually intact, is measuring tumor uptake of $^{89}\text{Zr}$-labeled bevacizumab by PET (El-Khouly et al., 2017; Jansen et al., 2017). This strategy could be applied to patients with TBM to study if this (or another) drug is indeed delivered to compact granulomas or inflammatory foci during TBM.

In conclusion, the combination of the zebrafish-\textit{M. marinum} model and clinical studies can help us to explain variation in pathological and clinical presentation and might identify host and/or bacterial targets for preventive or therapeutic approaches. The zebrafish model is instrumental in understanding the fundamental mechanisms explaining the molecular interactions leading to TBM. Advanced knowledge of genetic variation in TBM patients can direct future studies that can extent into the development of individualized therapy and improve outcome for the individual TBM patient.
REFERENCES


