Functions of leptin in tuberculosis and diabetes: multi-omics studies across species

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Chapter 6

General discussion and future perspectives

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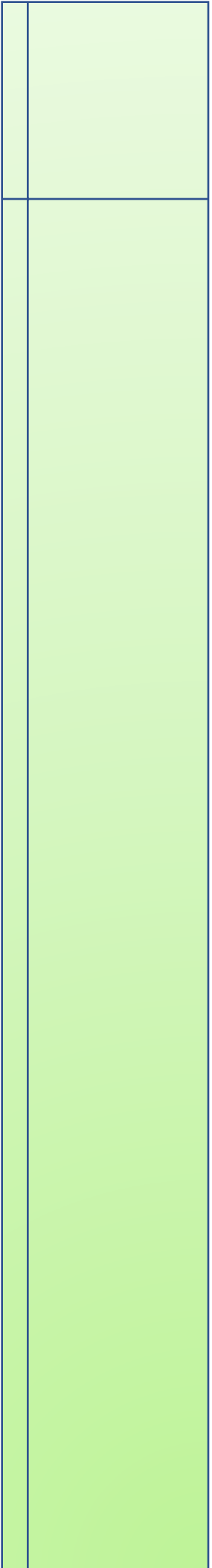

Zebrafish models for metabolic diseases

The zebrafish (Danio rerio) is an emerging model organism for studying acquired diseases including cancer and microbial infections, as well as metabolic diseases as it has many advantages [1, 2]. Zebrafish are genetically tractable and with high frequency can produce large numbers of larvae that are optically transparent which allows their real time imaging for tracking pathogenesis of diseases [3]. Zebrafish share approximately 70% genetic similarity with human beings [4, 5] and the basic cellular processes and molecular functions are highly conserved between mammals and zebrafish [6]. The growing availability of transgenic lines with fluorescent labels for instance in immune cells makes it possible to non-invasively and directly visualize the process of pathogenesis in the transparent zebrafish larvae [7]. Versatile zebrafish models for studying tuberculosis (TB) have been developed and are commonly used [8, 9]. In Figure 2 of **Chapter 1**, it is shown that *M. marinum* infection in zebrafish embryos results in characteristic necrotic granulomas, which recapitulates the process leading to human TB. Furthermore, the application of automatic robotic injection of *M. marinum* pathogens into the zebrafish yolk with a speed of up to 1000 embryos within 20 minutes at very high accuracy, contributes to a high-throughput level of screening [10-12]. The availability of a collection of large numbers of zebrafish samples upon infection also compensates for the relatively low sensitivity of NMR measurements. Therefore, the zebrafish model for TB continues to yield important insights into the discovery of new biomarkers for TB, early diagnosis of the disease and screening for new therapeutic treatments.

Rodent animal models with leptin or leptin receptor deficiency are well established and widely used for studies of obesity and diabetes mellitus (DM) [13]. Leptin and leptin receptor are highly conserved in fish and mammals [14, 15]. In the research described in **Chapter 3**, a *lepb* mutant zebrafish line is generated using CRISPR-CAS technology. The study of this zebrafish line shows that leptin deficiency in adult zebrafish at 1.5-year-old age leads to the development of DM and early signs of diabetic nephropathy as well as a significant accumulation of visceral fat measured by magnetic resonance imaging (MRI) [16]. A recent published paper show that no obesity is found in *lepr* and *lepa* zebrafish mutants compared to their respective controls [17]. The controversial results observed by different groups might be explained by different breeding schemes [17-20]. Wild type and *lep* or *lepr* mutant zebrafish raised together in the same tank show no significant differences in growth phenotypes [18, 20]. However, significant growth differences between genotypes are observed when they are raised in separate tanks despite the absence of consistent obesity phenotype [17]. This might be due to the differences in terms of food competition, background variation, or stress sensitivity between genotypes when different breeding schemes are used [17, 21]. It is also possible that during breeding, compensatory genetic mechanisms have led to differences in phenotypes. Such compensatory mechanisms have been reported to occur in various zebrafish mutant lines, although the molecular basis for these mechanisms is still not fully

understood [22]. Considering the lack of knowledge of function of the duplication of the leptin genes in zebrafish, the occurrence of compensatory mechanisms in *lepa* or *lepb* mutants is difficult to exclude. It is therefore highly interesting to generate *lepa* and *lepb* double mutants in future research. Furthermore, it is recommended that the various leptin mutants generated in different laboratories are exchanged between laboratories and subjected to comparative studies.

Conserved metabolic changes resulting from tuberculosis and leptin deficiency across species

In this thesis we have shown that metabolic alterations resulting from both TB and leptin mutation are highly conserved across mammals and non-mammals. It gives support for the hypothesis that there is a common molecular mechanism underlying wasting syndrome in all vertebrates. In **Chapter 2**, it is shown that a metabolic reprogramming processes during TB infection are conserved in humans, mice and zebrafish larvae. In **Chapter 4**, it is shown that leptin deficiency in adult mice and larval zebrafish leads to highly similar metabolic alterations in amino acid levels as well as in regulation of genes involved in the proteolysis and arachidonic acid pathways. In **Chapter 5,** it is shown that leptin mutation and mycobacterial infection lead to a similar metabolic syndrome in mice as well as in zebrafish larvae. The metabolic changes resulting from both TB and leptin mutation between different species are very striking considering the variety of differences between the analyzed samples. (1) Human, mice and zebrafish are very diverse examples of the vertebrate subphylum, e.g., metabolic rate, body size, body temperature and examined life stages vary greatly. (2) Samples of blood or body tissue, in the case of the human and mice experiments, are compared with the entire organism in the case of zebrafish larvae. (3) The environmental conditions are different in the three species. (4) Metabolic profiles are detected by different metabolomics technologies with different sample handling. (5) The genetic variation within the studied populations is highly diverse in zebrafish test samples, whereas a highly inbred population is used in the case of mice. (6) Since zebrafish larvae are not yet fed during the timeframe of the experiments, but solely use their yolk for nutrition, this shows that differences in food intake are not involved in the observed metabolic changes. The metabolic similarities, in spite of the many differences in the analyzed samples listed above, give important information and many new insights. Comparative analyses between mammals and non-mammals at metabolomics and transcriptomics levels provide essential information for further biological studies of tuberculosis and diabetes and drug engineering. The metabolic similarities and conserved pathways found in metabolic diseases among different species yield advances in various aspects. Firstly, it can give new insights in the evolution of controlling mechanisms of metabolism in vertebrates; Secondly, it offers new possibilities to study the mechanisms of metabolic alterations in the zebrafish larval model system; Thirdly, it shows the translatability of biomarkers from zebrafish and rodents to human diseases.

The role of leptin in tuberculosis

Tuberculosis patients usually suffer from anorexia and malnutrition, resulting in severe weight loss including extremely consumption of fat and muscle tissues [23]. Leptin is a key regulator of body weight, energy balance and immunity [24], therefore it might also play an essential role to defense against *Mtb* infection. Plasma leptin level is found to be lower in TB patients than in healthy controls [25-27]. Its concentration is increased after anti-TB treatment [28]. In contrast, leptin level is also found to be higher in TB patients than in controls [29]. To investigate the role of leptin in defense against mycobacteria infection, in **Chapter 5**, we look into the effects of mycobacterial infection in leptin deficient *lepb*$^{-/-}$ mutant zebrafish larvae and *ob/ob* mutant mice. Higher bacterial loads are observed in the *lepb*$^{-/-}$ zebrafish larvae as well as in the lungs of *ob/ob* mice upon mycobacteria infection, compared to the respective wild type infected controls. This observation is consistent with infection studies in *ob/ob* and *db/db* mutant mice [30, 31]. It is reported that leptin-deficiency is correlated with dysregulation of cytokines secretion, which increases susceptibility to infectious diseases including TB [32, 33]. However, in both species, the decrease in the levels of metabolites is not getting more pronounced when the leptin mutant is infected by mycobacteria. Therefore, we conclude that leptin and mycobacterial infection are non-synergistically controlling metabolism, but lead to a similar metabolic reprogramming. This is the consequence of many of the metabolite levels decreasing in response to infection in the wild type are already decreased in the absence of infection in the mutant compared to the wild type. The fact that the effect of infection by mycobacteria on metabolism is similar to that of a mutation in the leptin gene could be used as an argument that the bacteria are not directly responsible for the observed metabolic changes, but that their effect is indirect via the host system. Apparently, the wasting syndrome that is the result of a mutation of the leptin gene can't be further aggravated by consumption of metabolites by mycobacteria after infection of the leptin mutant.

The potential utility of leptin deficient zebrafish for gestational diabetes mellitus studies

In **Chapter 4**, it is shown that leptin deficiency in adult mice and zebrafish larvae lead to similar metabolite changes. The similarity of metabolic changes resulting from leptin deficiency between blood of adult mice and entire zebrafish larvae provides the potential to use common metabolites as biomarkers. These biomarkers could be indicators for the onset of diabetes in adults but also in their offspring, providing prognostic markers for the early identification of the risks of gestational diabetes mellitus (GDM). In this respect, it can be expected that the metabolic state of the parents can even influence the development of the second-generation offspring since the germ line cells are already developed before birth and at the larval stages in fish. We are planning to investigate this in more details in zebrafish, for instance by paying attention to epigenetic factors that could play a role in maternal or paternal influences on the offspring in the first and second generation. To

investigate the role of the age of diabetic parents on development of their offspring, we are currently analysing RNAseq data from the 5dpf offspring of different ages (0.5, 1 and 1.5 years old) of adult *lepb* mutant zebrafish and their wild type siblings. In addition, to see if there are gene expression differences caused by two *lepb* gene deletions (7bp and 8bp), RNAseq data from 5dpf offspring of *lepb*7 and *lepb*8 at 1.5-year-old will be investigated as well. Moreover, the offspring and muscle tissues collected from 2-year-old *lepb*7 and *lepb*8 adult zebrafish are currently sequenced by bulk RNA barcoding and sequencing (BRB-seq). BRB-seq is a novel high-throughput transcriptomics sequencing approach which is quicker and 20 times cheaper than conventional commercial RNAseq technologies [34, 35]. By studying the correlation of transcriptome changes caused by leptin mutation in adult zebrafish and their offspring, we aim to obtain more knowledge of parental effects of leptin deficiency on their off-spring and thereby provide new insights in the mechanisms underlying GDM.

Perspectives for future studies

The application of PINTA and μMRI in zebrafish

NMR spectroscopic analysis of biological samples often makes use of isotopic tracers [36, 37]. For instance, Perry et al. reported a method called positional isotopomer Nuclear Magnetic Resonance (NMR) tracer analysis (PINTA) [38]. This method allows non-invasively to model metabolism *in vivo* [38, 39] by combining NMR and gas chromatography-mass spectrometry (GC-MS) analysis of plasma following an infusion of [3-^{13}C] lactate and glucose tracer [38]. This method can be used for instance to examine the role of hepatic glucose, fat and amino acid metabolism in a physiological state in humans as well as in animal models. In a future study, we are interested in applying the PINTA approach for the real time investigation of hepatic metabolism in zebrafish. The use to adult zebrafish is relatively difficult due to ethic issues. Zebrafish larvae might be suitable as an alternative model by injection of [3-^{13}C] lactate and glucose tracer into blood vessels and then subsequent analysis by the combination of NMR and GC-MS.

In addition, magnetic resonance microimaging (μMRI) has been reported to be useful for imaging of intact zebrafish. It was used to detect tumors in live adult zebrafish despite the very small size of the cellular structures [40]. We are planning to develop an application of the μMRI technique to image younger zebrafish even at larval stage. For this, the micro-coil technology reported by Schadewijk et al. can be employed [41]. Using such technology it should be possible to image noninvasively the fat distribution and muscle volumes *in vivo*. This would allow us to investigate which tissues are affected by wasting syndrome at an early stage of zebrafish development and also open path for longitudinal MRI studies from larvae till adult stage. Furthermore, chemical shift imaging and localized MR spectroscopy at larval stage can provide the possibility to map the distribution of various metabolites *in vivo*.

Wasting analysis in zebrafish larvae

In **Chapter 4**, it is shown that the level of many metabolites in leptin mutant zebrafish larvae is decreased compared to the wild type siblings. The metabolic changes are similar with what we observed in leptin signalling-deficient mice which exhibit muscle loss and muscle weakness [42]. In future studies, we are interested in whether there is muscle wasting in the early development of *lepb* mutant zebrafish larvae. To study this, we can use the vertebrate automated screening technology (VAST) bioimager platform that has been developed and established for advanced volume imaging by Guo et al [43]. It allows to load zebrafish larvae automatically into the system and it enables 360 degrees rotation of zebrafish larvae during imaging [44, 45]. Due to the optical transparency of zebrafish larvae, most zebrafish organs including muscle can be visualized three-dimensionally. Bright field as well as fluorescent images of zebrafish larvae can be acquired. Muscles from zebrafish larvae can for instance be labelled using rhodamine-phalloidin. By reconstruction of fluorescent muscle images from different orientations of the larvae, muscle volume as well as the ratio of muscle and body volume can be determined. We want to use this method for high throughput measurements of muscle wasting during development in mycobacterial-infected wild type and leptin mutant zebrafish larvae.

Analysis of fatty acids in zebrafish larvae

Unpublished data from our laboratory show that *lepb* mutant zebrafish embryos display more fat vesicles than the wild type siblings at around 9 hours post fertilization detected by electron microscopy. Furthermore, oil red staining shows differences between 5dpf *lepb* mutant and wild type zebrafish larvae. We are investigating the amount of fat and fat distribution resulting from leptin mutation in more details by testing different time points of larval development. Furthermore, in **Chapter 4**, it is shown that the arachidonic acid pathway is enriched both in the transcriptomes of heads of *ob/ob* mice and *lepb* mutant zebrafish larvae. To further study this, a robust and sensitive targeted analysis platform: high performance liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS) using a dynamic multiple reaction monitoring (dMRM) mode, is available at Leiden [46-48]. The dMRM mode has short retention time windows and is highly sensitive [46]. This platform allows the quantitative measurements of arachidonic acid and its downstream derived eicosanoids down to nanomolar levels [46]. The application of this robust platform to the zebrafish model systems used in this thesis will provide important information on the role of eicosanoid signalling in the inflammatory processes underlying diabetes and mycobacterial infection.

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