Chapter 7

General discussion and summary

Thanks to all the great medical inventions in the last couple of centuries the life expectancy of the human population is significantly increasing. By the year 2050 twenty-two percent of the human population will be older than the age of 60. This puts great pressure on the health care, since this group will most likely need medical care at a certain point due to cancer, diabetes, osteoporosis, heart diseases or any other kind of age-related disease. However, there can still be great improvements achieved in the field of medical instruments and implants. Although there are already many implants or medical tools available, there is still a risk of infection, related to these biomaterials. One of the main priorities during development is the functionality of the material, such as a titanium hip that lasts a long time. However, biocompatibility is also a factor of great importance. Testing biocompatibility of new materials in early stages of development could save time and money. However choosing the right model for the research question can be difficult. Does the data from cell and tissues culture provide sufficient information or does it lack the complex networks that you can find in animal models, and which animal represents the human situation the best? At the same time the requirements for the laboratories and facilities are also playing a very important role, since some pathogens or animals can be examined in biosafety level 1 laboratories where other are restricted to biosafety level 3 or 4 laboratories.

Development and optimization of high-throughput zebrafish techniques

The work in this thesis uses zebrafish larvae as a test organism for studying various infectious diseases. Most of the advantages of using the zebrafish larvae as a model are already discussed in detail in the previous chapters. One of these advantages and the focus of this thesis is that zebrafish eggs and larvae are ideal for high-throughput screening. We analysed which developmental stage is optimal for spreading of bacterial though the body after yolk injection with 2 types of bacteria, namely *Staphylococcus epidermidis* and *Mycobacterium marinum*. We also compared the outcome with other injection methods like injecting into the caudal vein at 1 day post fertilization (dpf). We determined that injection into the yolk with *S. epidermidis* and *M. marinum* between the 16-128 cell stage results in reproducible infections with spreading of the bacteria into the tissues of the embryo at later stages. In contrast, injection before the 16 cell stage leads to a high mortality and injecting after the 128 cell stage does not lead to a representative infection. The more virulent *M. marinum* M strain does not even give a representative infection after the 64 cell stage (**chapter 3**).

The early developing zebrafish egg is ideal to automatically inject into the yolk, and we showed that this could be performed with RNA, DNA, bacteria or cancer cells (**chapter 2**). The automated micro-injector allowed us to inject up to 2000 eggs per hour in a consistent manner into the yolk of a developing zebrafish egg. This kind of specifications cannot be reproduced with manual injection techniques. Using this kind of automated micro-injector yields therefore a strongly increased number of samples that can be used for compound screening or testing other biological questions, and with that reduces valuable research time.

However handling these amounts of samples also requires fast and efficient analysis techniques. We made use of a model of flow-cytometry that can handle extraordinary large particle sizes (called COPAS XL), which could analyse and sort large amounts of infected larvae (2000/30min), without harming the larvae. Therefore this analysis could be performed daily to monitor the fluorescence signal of the bacterial burden in large groups of infected larvae (**chapter 4**). The only drawback of performing such flow-cytometry is the low resolution. However, to overcome this problem we also implemented a medium throughput, high resolution method. Therefore, we used the Vertebrate Automated Screening Technique (VAST BioImager), that automatically loads and positions the larvae to predefined settings. The imaging was performed using a confocal laser scanning microscope (CLSM) with an additional colour camera (**chapter 3**).

Using this high-throughput pipeline we were able to understand the pathogenesis of *S. epidermidis* in zebrafish larvae better. We performed intensive microscopy, flow cytometry and transcriptome analysis, on the infection process of *S. epidermidis* in zebrafish larvae. The use of the COPAS XL proved to be accurate to analyse the bacterial burden without homogenizing and plating the embryos. Secondly the use of micro-array analysis led to detailed host transcriptome expression, which was replaced by RNA deep sequencing (RNAseq) within the project.

Transcriptome analysis

Although the micro-array approach provides useful expression patterns of the transcriptome, the method of detection is rather biased. Micro-arrays use labelled probes for the detection which means that if a probe is not present on the chip it will not be found. RNAseq on the other hand counts short reads based on a reference genome as illustrated in Figure 1. This means that also expression of non-annotated sequences can be discovered and the analysis can be repeated when updated genomes are released. We chose to use the RNAseq analysis method using Illumina sequencing which is based on the sequencing of cDNA that is de novo replicated on flow cells. However, there are also other options available for the analysis of differential expression of RNA such as Ion Torrent sequencing, which also measures cDNA instead of RNA but on a semiconductor chip. In this technology small fragments bind to specific beads from where hydrogen ions will be released when a nucleotide binds to the fragment. This leads to changes of the pH that can be measured with a voltage meter. These changes in voltage can therefore be used to read the sequence. Another option that might be used in the near future is direct sequencing of RNA using the MinION device. This is based on consumable flow cells with nanopores that can sense single molecules. This little device with the size of a USB-stick can be used for DNA sequencing but could, theoretically, also be used also for RNA and protein sequencing. However, the nanopore technology is still under development and although it offers good perspectives for further decreasing cost of sequencing its suitability for direct RNA sequencing has still not been demonstrated.

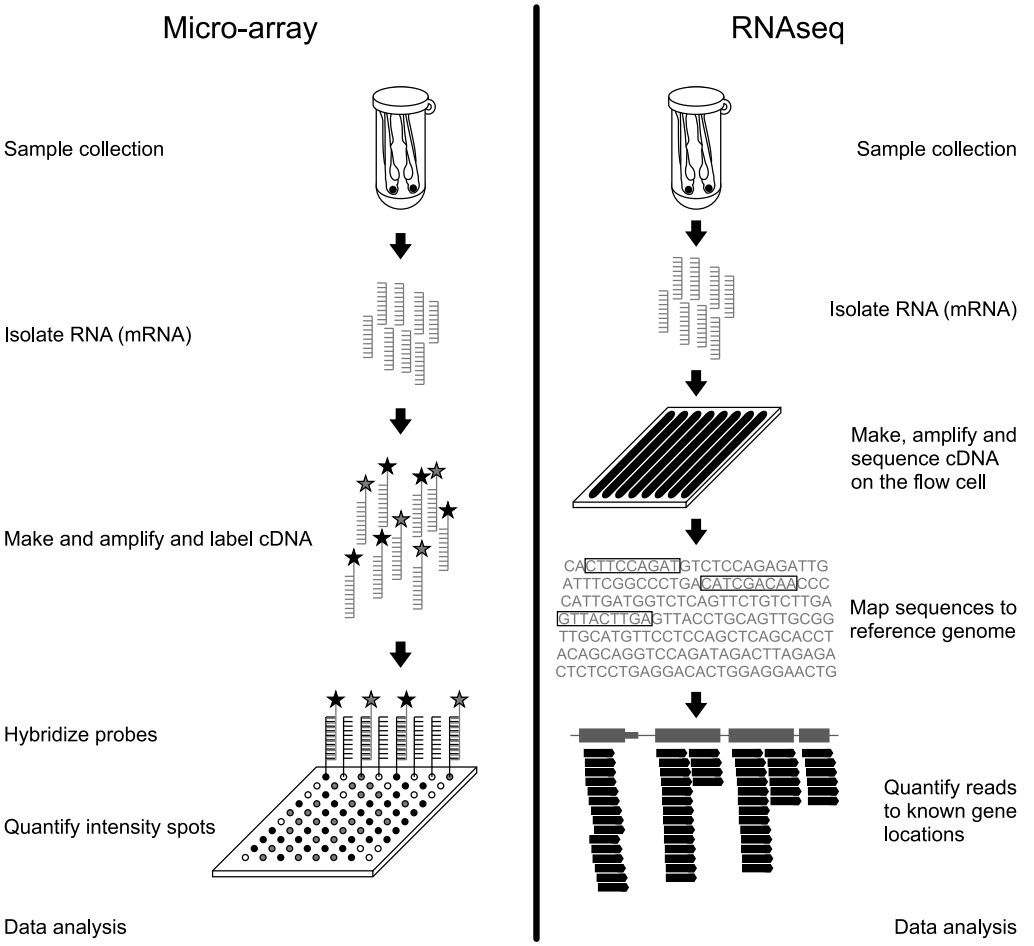

Figure 1: The differential expression analysis methods. The left side shows the micro-array technique with hybridization of the probes on the chip. Only targets present on the chip be found, and due to multiple probes of a target to improve sensitivity, this will reduce the total amount of total targets on the chip. The right side shows the RNAseq technique, which uses short reads to quantify the expression levels. Therefore unknown sequences can be found and the analysis can be repeated with newer and updated versions of the reference genome.

A possible draw-back of RNAseq technology is that the analysis of sequence data requires some dedicated bioinformatics skills that are not always available in biology institutes. A possible solution would be to outsource the bioinformatic analyses to companies but this is currently very costly. For that reason we collaborated with bioinformaticians and statisticians to design a platform for fast and user friendly analysis of large RNAseq data files. This resulted in the design of the GeneTiles software package (**chapter 5**), which combines all the programs needed for basic RNAseq analysis such as Bowtie2, Samtools, the 'R' statistical package, DESeq, DEXSeq, HTSeq and pysam. All these programs run in a pipeline in a server based environment, which can be accessed from every computer with operations systems such as Windows and Linux with an internet connection. One of the advantages of having the analysis run on a server is that there is no need any more

for powerful computers in the laboratory. To offer a complete package that includes the entire analysis and direct visualization, multiple options are implemented, such as differential expression sorting on ratio, *P*-value, adjusted *P*-value, differential splicing or just chromosome view. In addition to this we also included all available biological pathways from Wikipathways. This allows quicker and better interpretation of the expression data in a more complex manner. As result of this improved RNAseq analysis method we found a gene that was differentially spliced under infectious conditions, namely glucagon a (*gcga*).

Metabolic changes upon infection

Another finding was the high induction of the leptin b (*lepb*) gene in RNAseq data both *S. epidermidis* and *M. marinum* infected embryos. This was not discovered earlier since the *lepb* probe was not present on the micro-array. The leptin hormone is normally produced by fat cells, which regulate the fat balance in the human body. If a person consumes food, a signal is given to the hypothalamus, which then regulates the appetite, and prevents from overeating. However, if a person continuously overeats, this can lead to leptin resistance. This means that the leptin signal does not arrive at the hypothalamus and that there is no feedback signal that prevents overeating. Therefore people will eat more than they need and gain weight with obesity as a result (Figure 2). This can in turn also lead to continuous production of too high levels of insulin leading to insulin resistance. Insulin resistance is the hall mark of diabetes type 2 that results in chronic problems in diminishing blood glucose levels (leading to hyperglycaemia), and a lack of conversion of glucose to glycogen in tissues. Due to the high glucose levels in the circulation, people will also get more susceptible to heart diseases, stroke, blindness and kidney failure.

Since the leptin gene is a very interesting gene which is already linked to multiple metabolic and immune functions, and *lepb* was the highest induced gene upon infection with *M. marinum* and *S. epidermidis* we designed a morpholino to knock down the *lepb* gene functionality in the early development of the zebrafish embryo. Preliminary results suggested an induced bacterial burden in the *lepb* morphants, which indicates that *lepb* could have a role upon a *M. marinum* infection in zebrafish larvae. This prompted us to perform mass spectrometry on these *lepb* morphants with and without a *M. marinum* infection versus wild type controls. We found metabolites associated with the wasting syndrome (R. Marín-Juez, unpublished results) to be no longer influenced by infection in the leptin knockdown situation. The results therefore showed that leptin is an important player in the wasting syndrome caused by infection. This could indicate that there is cross regulation between nutritional status of the host and the immune response against *M. marinum*. This could also be a possible explanation of acute weight loss due to TB, since appetite is supressed by high leptin levels, due to infection. At later stages of infection by TB bacteria one could speculate that leptin resistance in the hypothalamus might occur, leading to a complex situation where a return of appetite in combination with wasting might lead to a new stage of tuberculosis disease progression.

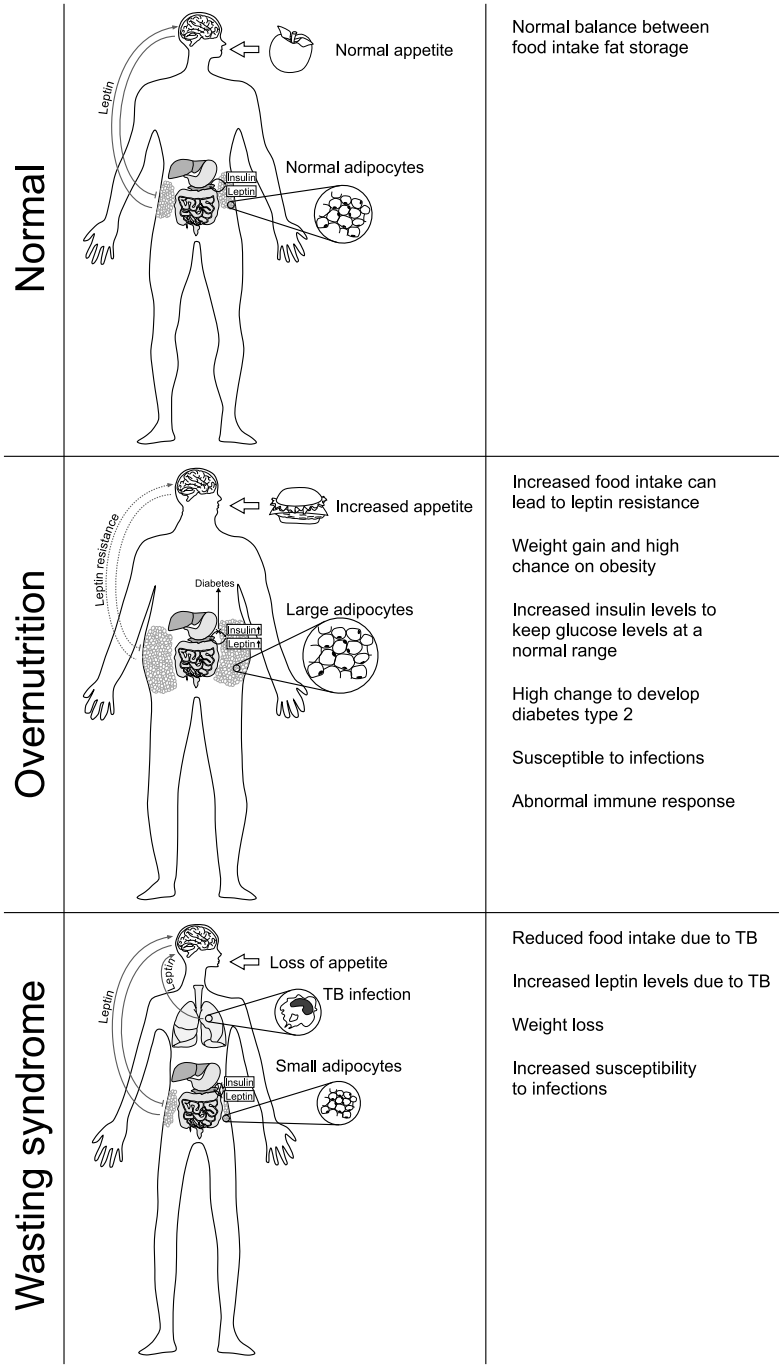

Figure 2: Functions of the leptin gene. Under normal conditions leptin regulates appetite and prevents from overeating. When a person overeats, the adipocytes will increase and the chance of leptin resistance occurs. This leads to uncontrolled appetite and chances of developing diabetes type 2. Leptin is also increased in TB infected people, which could suppress appetite with weight loss as result. This figure is based on Amitani et al. Front Neureosci. 2013 and Matarese et al. J Immunol. 2005.

Biomaterial-associated infection

As described above, we developed many different injection and implantation techniques for biomaterial-associated infection in zebrafish larvae (**chapter 6**). However, reproducibly implanting biomaterials into zebrafish embryos appeared to be more difficult than expected. Injection of polystyrene beads into the tail muscle proved to be rather labour intensive, whereas injection into the yolk did not lead to high frequencies of distributed biomaterials. We did find a size dependent distribution of beads ranging from 70 nm to 15 µm. The smaller sized beads did distribute more than the larger ones. We have not found the mechanism yet that explains the distribution of these particles, but hope to elucidate this in future work. The implantation of beads could in combination with the infection model of *S. epidermidis* in zebrafish as described in chapter 3, 4 and 5, be an important addition to the already known mammalian research models on biomaterial-associated infection.

Conclusion

Infectious diseases are everywhere around us, and we need to keep improving our knowledge about our defence mechanisms and the evasion strategies of the pathogens. The work in this thesis describes multiple techniques that can contribute to fast screening methods in order to come up with new strategies against infectious diseases. The use of automated micro-injectors in combination with large flow cytometers and automated microscopy has shown added value (**chapters 2, 3 & 4**) for research questions about (opportunistic) pathogens. The collaboration between scientists of different research areas has proved to be very successful in the development of an easy to use analysis platform for the analysis of RNAseq data (**chapter 5**). This has led to very detailed description of host molecular expression patterns following infection by these pathogens. This could be used to gain more insight in how biomaterials behave in a host environment in the presence or absence of infection (**chapter 6**). All together this has led this to a variety of research methods that can be used for studies of infections caused by many bacteria such as *S. epidermidis* and *M. marinum* described in this thesis, but, also by other microbes, such as fungi.