Chapter 1

Outline of the thesis

Public health and infectious diseases

Biomaterial associated infections

Between the year 2000 and 2050 the percentage of people over the age of 60 worldwide is expected to rise from 11% to 22%. The total number of this population is estimated to grow to 2 billion in this same period. Eighty percent of these older people will live in low and middle income countries. This particular group of in low and middle income countries will have a 3 times higher chance of lower quality of life due to noncommunicable diseases such as cancer, diabetes, osteoporosis and heart diseases [1-5]. This group of rather vulnerable people will probably receive medical treatment once or more in their old age. With many procedures patients will get in contact with biomaterials, ranging from infusion needles to implants, such as pacemakers or artificial joints. These biomaterials as shown in Figure 1 are often associated with infections by the skin bacterium *Staphylococcus epidermidis* [6-12]. Additionally, infections with (multi-drug resistant) *Staphylococcus aureus* strains are a common problem in hospitals. The first publications about biomaterial-associated infections (BAI) go back to the early 1950's by Elek et al. [13]. They discovered higher infection rates of *S. aureus* inoculum on a soaked suture wire compared to standard inoculum in human volunteers. In more recent studies, the percentages of infection related to implantation are approximately between 1.2 and 53% [14-18]. Since materials are being implanted daily in many patients worldwide as shown in Table 1, the total number of resulting infections is very large. There would not be a problem if these infections were easy to treat, however

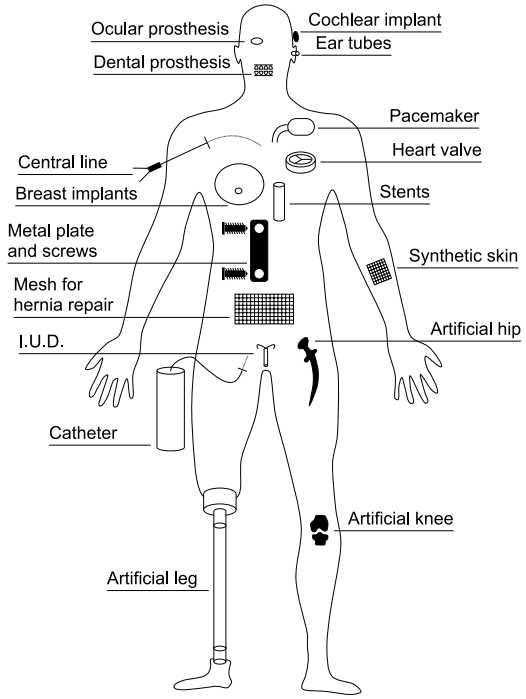

Figure 1: Overview of commonly implanted biomaterials and medical devices. This figure illustrated the variety of medical implants being used these days.

Table 1: List with 11 most implanted medical devices in the U.S.A. (data adapted from 24/7 Wallst 2011-07-11) [23].

#	Description	Number of procedures	Total cost annual	Average cost per procedure
1	Artificial eye lenses	2.582 million	$8-10 billion	$3.200-4.500
2	Ear tubes	715.000	$1-2 billion	$1.000-4.500
3	Coronary stents	560.000	$7,5 billion	$13.000
4	Artificial knees	543.000	$12 billion	$22.000
5	Metal pins, screws, plates and rods	453.000	$4,5 billion	$2.000-20.000
6	Intra-Uterine Devices	425.000	$340 million	$800
7	Spine screws, rods and artificial discs	413.000	$10 million	$25.000
8	Breast implants	366.000	$992 million	$3.351
9	Pacemakers	235.567	$4,5 billion	$20.000
10	Artificial hips	230.000	$10,5 billion	$45.000
11	Implantable Cardioverter Defibrillators	133.262	$5,5 billion	$40.000

S. epidermidis will adhere to the implanted material where it forms a biofilm. From there it will also infect the surrounding tissue and host cells, even after the material has been removed as has been shown in mice [10, 19]. This makes treatment of BAI more difficult, since some antibiotics like vancomycin cannot penetrate the infected tissue efficiently [20]. This can result in many complications with in the worst case scenario the necessity of the chirurgical removal of the implant. To prevent BAI we hope to find new molecular factors that could help understand how bacteria growing at or near implanted materials interact with the host. Although most research regarding BAI is performed with mammalian models such as mice or goats [21, 22], we used the zebrafish larvae as model as described in more detail below.

Tuberculosis and the link with metabolic syndromes

An infectious disease not related with biomaterials but forming another major societal problem is tuberculosis (TB), which is caused by *Mycobacterium tuberculosis* (Mtb). Mtb is one of the most successful human pathogens and is capable of establishing infection despite the host innate and adaptive immune responses. Each year 9 million people develop TB and over 1,5 million die from it. A third of the global human population is estimated to be latently infected, and has a 5-10% lifetime risk of developing TB reactivation disease [24]. Therefore finding better treatments and diagnostic tools for this disease is essential. With the upcoming antibiotic resistance for the first line antibiotics including Isoniazid (1952), Rifampicin (1966), Pyrazinamide (1952) and Ethambutol (1961), there is a great need for new antibiotics [25]. The World

Health Organization (WHO) reported that already over 300.000 people were reported with multi drug-resistant tuberculosis (MDR-TB) in 2013. Extensively drug-resistant tuberculosis (XDR-TB), bacteria are resistant also to second line drugs, and has been reported by 100 countries in 2013. It is estimated that 9% of the people diagnosed with MDR-TB have in fact XDR-TB [26].

Although *in vitro* models can provide detailed information of how TB bacteria are taken up and survive in macrophages, it does not reflect the infection *in vivo*. Unfortunately mice have limitations as a model to study the pathogenesis of TB, since they do not form the characteristic granulomas with necrotic centres after infection. Guinea pigs and rabbits do show the granuloma formation, however they are relatively expensive and do not allow advanced genetic approaches, real time imaging or high-throughput screening. The zebrafish larvae infection system (this thesis) can with the use of the natural pathogen *M. marinum*, a close relative of Mtb, model the early steps in human TB disease progression very well. After infection of zebrafish embryos with mycobacteria the innate immune cells will form granulomas, with in later stages of development also the formation of necrotic centres [27]. As outlined below zebrafish larvae allow the use of advanced genetic and real time imaging technologies and are excellently suited for high-throughput screening.

Although TB is already a major health issue and difficult to treat, it becomes more problematic when people are also infected with the human immunodeficiency virus (HIV) [28]. The WHO estimated that people have a 26 to 31 times higher change to develop TB when already diagnosed with HIV [29].

Unfortunately not only HIV is a great risk in combination with TB, also obesity is linked to TB [30-32]. Obesity and overweight are defined as abnormal fat accumulation which is a great health risk. It is also a large risk factor for example for diabetes, cardiovascular diseases and cancer [33, 34]. Although commonly perceived as a problem in high income countries, its incidence is now also rising in low- and middle-income countries. In 2008 more than 1.4 billion adults globally were overweight and more than half a billion were obese. Obesity among children is ever so problematic since already 42 million children worldwide are overweight who will likely become obese adults, and are more susceptible to diabetes and cardiovascular diseases [35]. Many treatments are known, however, due to ethnic backgrounds and different lifestyles globally, it is a challenge to develop new strategies to prevent obesity [36].

A particular gene that regulates appetite, called leptin, has been well described in the context of metabolic processes [37] and also functions in immunity and inflammation [38, 39]. Overnutrition and obesity are correlated with high concentrations of leptin. Having high levels of leptin can lead to leptin resistance. This in turn can lead to increased TNF-α production, altered T cell subset ratios and repressed T cell response [40], with as a result, higher incidence of infectious diseases. This suggests that leptin resistance would be disadvantageous in the context of TB treatment, and there are scientific papers that could confirm this statement [41, 42]. However there are also few papers indicating the opposite, that there is a positive effect on TB progression due

to obesity [43]. The role of leptin has been described in the context of TB and obesity [30] but clear evidence that obesity as a result of leptin resistance could lower the progression of TB has not been found yet.

Zebrafish as a model for innate immunity research of human diseases

The zebrafish finds it origin in the Himalayan region and it is a popular aquarium fish. The first scientific experiments with this organism date from the early 1970's by Dr. George Streisinger [29], who used it for studies of vertebrate development and genetics. Since then it showed to be a versatile model for studies of human diseases such as cancer, infectious diseases, cardiovascular disease, diabetes, haematopoiesis, and neural disorders [44-46]. Although zebrafish do not have lungs and are cold blooded they have high similarity with mammalians from a genetic and developmental perspective. Since the larvae are very small and transparent, they are easy to follow in time, also due their fast development. The first innate immune cells are present at 1 day post fertilization (dpf). One adult couple can produce up to 300 eggs per week and the larvae are therefore very well suited for high-throughput screens. The availability of many mutant and transgenic strains makes it possible for example to follow live fluorescently labelled macrophages or neutrophils interact with fluorescently labelled pathogens inside a larva. Because they are so small, housing costs per animal are low. Many tools are available to identify genes involved in specific biological processes, such as random mutagenesis or targeted mutagenesis using zinc-finger nucleases or the CRISPR-Cas system [47, 48]. Also, using a morpholino approach, which is a ~25 base artificial oligonucleotide, injected into the one cell stage, specific gene function can be knocked down for the first couple of days of embryonic development [49, 50]. Altogether, this has led to the common use of zebrafish larvae in biomedical research. In addition, the model is better suited for translational studies than invertebrate animals since over 80% of genes linked with human disease have at least one obvious zebrafish orthologue [51]. Therefore it holds a very strong position as a screening model with a high-throughput level in between cell and tissue culture systems and higher animal models.

Outline of the thesis

The work described in this thesis focusses on the establishment and improvement of automated and high-throughput techniques for immunological studies in zebrafish larvae. We studied the innate immune host response towards *S. epidermidis*, a normally innocent human skin bacterium that is one of the main causes of biomaterial-associated infections. To this end, we used transcriptome analysis tools such as micro-arrays and RNA deep sequencing (RNAseq). To compare the results of infection with *S.*

epidermidis with the response to a natural fish pathogen, we performed simultaneously experiments with *M. marinum*.

Chapter 2 describes a broad overview of all the applications of injecting automatically with a robotic micro-injector. These include the generation of transgenic animals by injecting DNA into the yolk of a one-cell stage fertilized egg, or the knockdown of particular genes by injection of morpholinos. Furthermore, injections of opportunistic or pathogenic bacteria as well human cancer cells are described in detail. Analyses of the large amounts of injected eggs are performed with either automated confocal laser scanning microscopy (CLSM) or high-throughput Complex Object Parametric Analysis and Sorting (COPAS XL).

Continuing on chapter 2 we optimized our high-throughput screening system using video recorded procedures as shown in **chapter 3**. Here we show in detail how multiple devices such as a large spawning tank, an automated micro-injector, a COPAS flow-cytometer, and a Vertebrate Automated Screening Technology (VAST BioImager) can yield a combination of a high-throughput low-resolution with a medium-throughput high-resolution screening technology. Furthermore we optimized the injection method by selecting the best developmental stage for early yolk injections with *S. epidermidis* and a highly and medium virulent *M. marinum* strain.

Chapter 4 is focused on the pathogenesis of *S. epidermidis* in zebrafish larvae. This was the first scientific report of injecting *S. epidermidis* into zebrafish larvae. We performed extensive imaging and automated the monitoring of bacterial burden progression in live larvae using the COPAS XL. It was found that injection of *S. epidermidis* into the caudal vein did not lead to an infection, however when injected into the yolk of a 16-128 cell stage embryo, we did manage to reproducibly create an infection spreading into the entire body of the larvae. Time course analysis of transcriptome host responses was performed using micro-arrays. This led to the discovery of a group of host genes that were differentially expressed after infection by *S. epidermidis* and not by *M. marinum*. The validation of the micro-array results was performed using a newer technique, the so called RNAseq. Since transcriptome analysis using micro-arrays and RNAseq can provide a lot of information on how the host reacts to an invasive agent such as a bacterium, it can reveal possible targets for new treatment strategies.

Although the RNAseq method is very promising, the analysis still relies on expensive 'black box' software packages or dedicated programming skills of the researcher. However the second option could be rather difficult for a biologist untrained in informatics. In **chapter 5** we describe the results of collaboration between bioinformaticians, statisticians and biologists. This led to a straight forward and easy to use software package called GeneTiles, for the analysis and visualization of large RNAseq datasets.

One of the main advantages is that is runs on a server with a user-friendly interface. This means that is can be accessed from computers with an internet connection and different operations systems such as Windows and Linux can be used. The implantation of additional software packages such as DEXSeq has led to the discovery of differentially expressed genes under infectious conditions. Also a direct visualization of the differential expression in a variety of biological pathways obtained from Wikipathways leads to a fast interpretation of functional data.

In **chapter 6** we present a time course analysis of the distribution of nanometer and micrometer sized polystyrene particles after zebrafish yolk injection. This could help us to develop a screening model for biomaterial-associated infection using zebrafish larvae. We determined the parameters for the diameter and shape of the particles that determine the possibility to inject these particles using glass micro capillaries into zebrafish larvae. The size is also very important for the distribution inside the larvae, since we found that the smaller sized particles spread more compared to the bigger particles.

In **chapter 7** the results are summarized and discussed in the context of possible applications of the results for biomedical purposes.

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