Annual Scientific Report 2013

Biomedical Primate Research Centre

COMMITTED TO HEALTH RESEARCH AND ALTERNATIVES

Letter from the Chairman of the Supervisory Board

Health and public health issues remain highly relevant for society. Consequently, containment and/or prevention of diseases are of utmost importance. Research at BPRC continues to focus on those health-related problems which are difficult to address without the use of non-human primates. On the other hand studies to explore suitable alternatives are also part of the research programme. This annual report offers a thorough in depth view on the efforts made by BPRC to unravel suitable solutions for a number of diseases. Apart from the major infectious diseases, including malaria, tuberculosis and HIV/AIDS, emerging virus diseases with a threat to human and veterinary public health, like Dengue virus and West Nile virus are studied as well. In the light of the ongoing risk of seasonal epidemics by influenza viruses interesting research on the development of a new vaccine concept has been performed. Chronic diseases like multiple sclerosis and rheumatoid arthritis continue to be a challenge to researchers. Non-human primates offer very valid models to understand the dynamics of the disease processes and to suggest options for treatment.

Basic studies on the immunological system remain necessary to make optimal use of the animal models. Furthermore, animal behaviour studies are essential to improve animal care and welfare at BPRC, a very important part of the quality of the research.

BPRC remains open to the public at large. Being a knowledge institute it is a prerequisite that the way research is being performed is shared with society.

The Supervisory Board is well aware of the professional attitude of everybody at BPRC which makes it possible to continue the urgently needed studies in the interest of society.The annual report is recommended to the general public, but also to professionals in health sciences and to policy makers who are offered a well balanced look into a research area with a great potential.

Professor Joost Ruitenberg

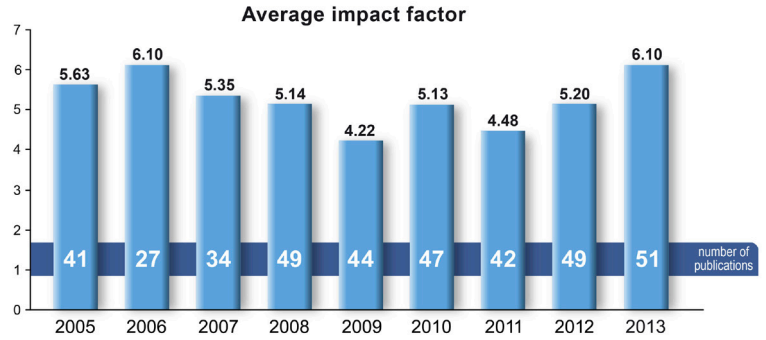
Chairman of the Supervisory Board

Message from the Director

Dutch law defines that biomedical research with animals is not allowed if alternative methods are available. Non-human primates are essential in biomedical research for an array of infectious and chronic diseases that are a burden to mankind, due to the fact that alternative methods are not available. For instance, in the case of diseases like Malaria, AIDS, Tuberculosis, Multiple sclerosis and Alzheimer there is a desperate need for the development of safe and effective vaccines, therapies or medicines. The immune system of humans and non-human primates share many similarities. As a consequence, many new medicines or vaccine candidates can only be tested for safety and efficacy in a proper non-human primate model, as those components are not reactive, for instance, in rodent species. Next to that, BPRC has a reputed research line based on the so called 3Rs principle (Reduction, Replacement and Refinement), which is in line with our societal Responsibility (fourth R). BPRC also puts a lot of effort in behavioural research in collaboration with the University of Utrecht.

In concert with our high research standards, the BPRC continues to improve its animal welfare issues, and much effort has been put in training animals. All animals are housed in pairs or larger social configurations in large indoor and/or outdoor facilities matching the best standards. The BPRC breeds its own animals and has become independent of importation.

The relevance of BPRC's research efforts is highlighted by the fact that our staff members published 51 manuscripts in peer-reviewed journals, with an average impact factor of 6.1. Many of these papers were published in high impact journals. For an overview, please consult the news section of our website (www.bprc.nl).



As can be seen later in the report, the year 2013 was closed with a small positive financial result. Finally, I would like to thank all the BPRC employees, but also all the other dedicated persons, who have helped us to maintain and improve BPRC's research and animal welfare standards.

Prof. dr. Ronald Bontrop
Director BPRC

Ethology

Aim of the ethological research

Like humans, primates are highly social animals. They spend their whole life in a group and their social behavior is varied and complex. Studies comparing different species indicate that social complexity has selected for relatively large brains. Moreover, a relatively large brain size has been connected to animal intelligence. However, how smart primates actually are and how they employ these capacities in their behavior is still an open question. The ethology research at the BPRC aims to address these questions with observational studies and non-invasive behavioral experiments.

Contribution to knowledge

The smartness of primates and their relationship to social behavior is explored in a research program that studies monkey social behavior and primate intelligence. Humans are part of the so-called ape lineage, that also includes apes like chimpanzees and gorillas. Less closely related to humans are monkeys, including baboons and macaques. The least related are prosimians, such as lemurs from Madagascar and nocturnal bush babies from Africa. It has been proposed that apes (e.g. chimpanzees) are smarter than monkeys (e.g. macaques) and that these are (again) smarter than prosimians. However, while apes are studied at numerous locations, research on the intelligence of monkeys is relatively scarce. Therefore, the lack of evidence on monkey-intelligence may also be due to the relative small research effort. Moreover, the evolution of human capacities can only be understood when also monkeys form part of the picture.

At the BPRC, this research focuses on monkeys, in particular on macaques. We study their social interactions and their social communication.

Why study non-human primates

Since primates are our closest living relatives, insight in their social behavior and intelligence provides crucial knowledge for understanding the evolution of human sociality and intelligence. In addition, such knowledge is crucial to the improvement of primate husbandry and housing in all captive settings.

Results in 2013

The output concerns scientific papers and reports in the popular press on primate social strategies, primate smartness and primate communication.

The social complexity of primate groups is evident in the differentiated relationships that members of primate groups maintain. Relationships with kin and friends involve by high rates of proximity and grooming. However, whether these relationships are actively maintained by the animals or are only side-effects of dominance interactions is unclear. We found that macaques maintain relationships over a long period. The longest stable relationship lasted at least 3.5 years. This is consistent with the idea that macaques maintain their social relationships by seeking particular social partners. Moreover, while dominant males would like to prevent subordinate males from mating, these subordinate males and also the females in the group seek mating opportunities. We showed that they enhance their options to mate, by showing sneaky mating behavior when dominant males are absent.

Humans can not only predict how others will behave, but also what others know. While at other institutes behavioral experiments with chimpanzees showed that they understand that another chimp can see them, monkeys failed at such tasks, indicating that apes are indeed smarter than monkeys. Our research counters this idea, by showing that macaques, a monkey species, do know whether a dominant can or cannot see food and adjust their behaviour accordingly.

Ethology

Animals communicate through facial expressions and vocalizations. We established in common marmosets the meaning of several facial expressions. These facial signals can invoke friendly behavior and animals can signal stress and fear resulting in a higher rate of affiliative behaviour. We could demonstrate that despite the fact that they live in small groups in trees, common marmosets use several visual expressions to communicate among each other.

Furthermore, research on primate vocalizations in collaboration with the Utrecht University showed that wild and zoo-housed orang-utans have flexibility in their call production. These apes can learn a new sound, namely to whistle, and can modify their sounds by using their hand or leaves. This indicates that some of the flexibility that is typical for human language is also present in apes.

The research has received attention in the popular press. In particular the sneaky mating behaviour in macaques and the signals in common marmosets were highlighted.

Animal Science

The main task of the animal science group is the provision of husbandry and optimal care for the animals and support for the research groups. In addition, the ASD plays an important role in the continuous further improvement of animal care and welfare at the BPRC.

Veterinary research

The veterinary group is involved in research to improve veterinary methods and increase animal welfare. Housing of animals in open settings, such as done at the BPRC, also increases the risk of infections, e.g. with parasites such as *Entamoeba spp.* and Giardia. These parasites can cause severe diarrhoea and treatment is then mandatory. In veterinary medicine, metronidazole is the most common treatment for these parasites. However, the administration of metronidazole in drinking water was not successful in rhesus monkeys due to low fluid intake attributable to unaccepted taste. Therefore another anti-amoebic medicine, clioquinol, was studied. Together with the Department of Pharmacy of the Veterinary Faculty in Utrecht a method was developed to dissolve this drug, which is not soluble in water. This medicated solution was well taken by the animals. Currently, the efficacy of this treatment in naturally infected animals is being evaluated. Other research parts included improved methods to allow acquisition of data without the need to handle the animals, such as by using biotelemetry.

To monitor inflammation in macaques more efficiently, we analysed together with the Department of Veterinary Clinical and Animal Science, University of Copenhagen, various methods to determine serum proteins and to correlate them with ongoing inflammatory events. Early detection of these compounds can be used to monitor the animals and provide early treatment when necessary.

Animal Training and Enrichment

To improve sampling using methods that disturb the animals as little as possible, BPRC investigators have implemented various animal training programs. In these programs the animals are trained to voluntarily cooperate within the specific studies. As partner within the EU-subsidised EUPRIM-Net program, various lectures have been prepared for training animal caretakers and active training programs are in place. The BPRC considers training and continuous improvement of the training methods essential.

In 2013 we studied the impact of various forms of enrichment in the experimental facilities on the animals. The aim of this study was to provide evidence-based selection of the most optimal enrichment for these animals. The data are currently analysed.

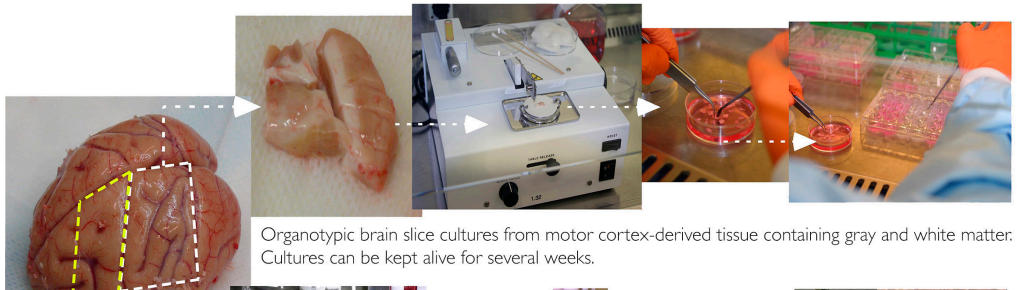
Alternatives for research in animals

Next to research for the benefit of human health, the second focus of research at the BPRC is the development of alternatives for animal experiments. These alternatives are developed along the guidelines of the three **R**'s of **R**efinement, **R**eduction and **R**eplacement. Efforts within the BPRC are organized at multiple levels. The three **R**'s are deeply embedded in the Animal Science Department and all research departments educate a minimum of one PhD student who will work on Alternatives. In addition, there is a separate Unit that is dedicated to the development of Alternatives for animal experiments.

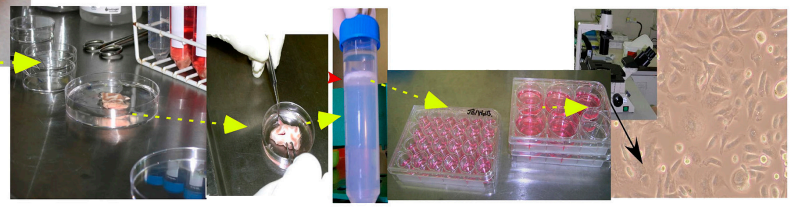
Organotypic and cell culture methods

A straightforward alternative for performing animal experiments is to try to step-by-step replace them by methods that can be performed in laboratory dishes (*in vitro*). Such *in vitro* methods can try to model an entire organ or can try to model specific cell types. At the BPRC we are setting up methods to model the liver, skin and brain. These organotypic culture methods are started from tissue material from deceased animals that would otherwise have been discarded. The material is carefully prepared, sliced and put into plastic culture dishes where it can be used for experiments.

In addition, we use the tissue to start primary cell cultures. Cultures of primary cells are much closer to normal cells than the more often used cell lines or tumour cells that can be kept in culture indefinitely. Primary cell cultures have a limited life span and should be started over and over again. Not all cell types can be easily cultivated in lab dishes, and in particular cells of organs like the brain are difficult to cultivate. Within the Unit Alternatives a large variety of primary cell culture methods has been developed and characterized. These methods enable researchers to test their research questions on relevant cell types before performing animal experiments. Such a *pre in vivo* test phase leads to a considerable reduction in the number of animal experiments. It is important to note that organotypic and primary cell cultures are always initiated from 'rest material' derived from donor animals that were euthanized as a result of e.g. other experiments. Thereby the end of one experiment is the beginning of another.



Organotypic brain slice cultures from motor cortex-derived tissue containing gray and white matter. Cultures can be kept alive for several weeks.



Dissociated primary single cell cultures from prefrontal white matter. Oligodendrocyte cultures are viable for 12~24h, microglia cultures for 7~10 days, astrocyte cultures for up to 10 passages (several months).

Last year new data with particular relevance for neuroinflammation were obtained demonstrating that cells from the brain respond differently than their counterparts in the blood. We have not only demonstrated that they respond differently, but also describe why they respond differently. Such insights are important when clinical therapies are developed that should work in the brain. In addition, a series of pilot experiments were performed to set up protocols for the cultivation of stem cells. Stem cells can be kept alive indefinitely and

can, in theory, be transformed into any cell type from the body by providing them with the right stimuli at the right time. This would open additional *in vitro* possibilities, especially for cell types like neurons that we are currently unable to grow as primary cells.

Refinement of experimental methods

Much Refinement is achieved by the Animal Science Department in the housing, training and daily lives of the animals. Refinement can also be achieved by optimisation of experimental procedures to reduce animal discomfort. One line of research with the Unit Alternatives aims to refine the use of adjuvants. Adjuvants are additives that are used in vaccines to enhance immune responses directed against pathogens or in experimental animal models for e.g. human autoimmune diseases. However, many potent adjuvants cause adverse effects. Most notable is the development of granulomatous skin lesions, causing various degrees of discomfort to non-human primates in biomedical experiments. There is therefore an urgent need for new and better adjuvants.

Recently it has become clear that the immune-enhancing effects of adjuvants can be attributed to activation of receptors of the innate immune system. Based on this knowledge we have engineered a series of luminescent cell lines and used these bioassays to screen new adjuvant candidates for their immune-enhancing effects. In addition, we have developed a 3D *in vitro* granuloma assay to screen adjuvant candidates for their potential to cause adverse effects. Combining these assays as a robust integrated test system has generated detailed information on a number of new candidate adjuvants. Last year we have finalised these *in vitro* screenings and we have selected several candidate adjuvants that will be tested in experimental models next year.

Communication, transparency and discussion

To enhance transparency the BPRC annually publishes a report on the use of experimental animals in the facility (www.bprc.nl/download/BPRC-PDKJ2012.pdf). Next to activities within the BPRC that are aimed at animal welfare, BPRC employees are also active in national (www.nkca.nl) and international initiatives (Expert working group 'Non-human primates in monoclonal antibody studies' organized by the UK national centre for **3R**s (www.nc3rs.org.uk/) to stimulate the use of alternatives.

Immunogenetics

The MHC in humans

It is well known that recipients of an organ transplant may reject the organ. To reduce the risk of this negative response, recipients and the candidate donors are tissue-typed, and the patient who matches best receives the available organ. In fact, tissue typing involves testing for the proteins of the Major Histocompatibility Complex (MHC), which in humans is named Human Leukocyte Antigen (HLA). MHC proteins are present on the cell-surface of particular body cells. The genes that encode these proteins are highly polymorphic, which means that they vary widely within the population. The MHC has not evolved to hamper the artificial process of organ transplantation. In the immune response, MHC proteins play a pivotal role in the recognition of foreign structures such as bacteria and viruses. The MHC proteins bind fragmented particles of microbial antigens, and present them to T lymphocytes, leading to specific immunological defence reactions. Particular variants of MHC genes (alleles) have been shown to be associated with susceptibility or resistance to infectious or autoimmune diseases.

MHC-typing in non-human primates

Since non-human primates are used as animal models to study immune-related diseases, their MHC genes are thoroughly investigated within the Department of Comparative Genetics and Refinement, with the aim of selecting the most suitable animals for certain types of investigations. Typing is based on DNA sequencing of the MHC genes. DNA, the chemical substance of which genes are composed, is a long string-like molecule formed by series of four different bases. The sequence of these bases in a particular MHC gene determines the structure of the encoded protein on the cell-surface, and hence the capacity to bind antigens and generate immunological defence reactions.

Recent investigations

Considerable research has been carried out in genetically characterizing the breeding colony of rhesus macaques at the BPRC. Almost 1400 animals have been tested for their most significant MHC genes, and 140 different haplotypes have been distinguished. A haplotype is defined as a combination of MHC genes on one chromosome, which are usually inherited "en bloc". An individual has two haplotypes, each inherited from one of the parents. All the data have been stored in a database, which minimises the need to obtain samples of blood or tissue from the animals.

MHC genes vary within the population, and polymorphisms are observed mostly in the part of the gene that encodes the antigen-binding region of the protein. The antigen-binding motifs for a few rhesus monkey genes have been explored, with the aim of contributing to a better understanding of T-cell-mediated immune responses.

The Department has recently started to investigate the MHC of the common marmoset (*Callithrix jacchus*). Some functional aspects of the MHC in these animals appear to differ substantially from those in humans. In humans the HLA-A, -B, and C proteins perform the classical antigen presentation function (activation of the immune defence), whereas HLA-G is expressed only in the placenta. This gene is thought to play an important role in protecting the foetus from potential hostile immune responses from the mother, directed against MHC polymorphisms inherited from the father. In the common marmoset, the most profound antigen presenting MHC protein is encoded by a gene, which is structurally related to HLA-G. These results demonstrate the plasticity of the MHC in primates.

Multiple sclerosis

What is MS?

Multiple sclerosis (MS) is a progressive neurological disease that affects exclusively the central nervous system, comprising brain and spinal cord (CNS). The cause of the disease is not known, but it is widely believed that in individuals with genetic susceptibility to MS, infection with certain viruses or bacteria triggers a complex pattern of immunological reactions against the CNS. The primary target of this attack is the myelin sheath, being a multi-lamellar structure that winds around nerve tracks (axons), produced by specialized cells of the CNS, called oligodendrocytes, (Figure 1). An intact myelin sheath protects axons against damage and ensures a rapid conduction of electrical pulses from nerve cells to the organ or tissue on which they project. The sheath contains multiple proteins, each with a different function, each of which can be a target of the immune attack. The focal infiltration of immune cells, such as B and T cells or macrophages, and immune factors, such as antibodies, from the blood into the CNS causes inflammation and destruction of myelin (demyelination); such demyelinated areas are called lesions. In the early phase of the disease inflammation can be suppressed by counter-regulatory immune mechanisms. Demyelination can be stopped and reversed by the formation of new myelin (re-myelination). The episodic flare and waning of immune activity underlies the typical relapsing-remitting (RR) course of early MS, where episodes of neurological function loss (relapse) alternate with recovery. Later in the disease recovery becomes incomplete and ultimately disappears, i.e. secondary progressive MS (SPMS). In a minority of patients (±15%) the disease is progressive from the onset, i.e. primary progressive MS. The reason of the transition from RR to SP MS is unknown and thus subject of intensive research.

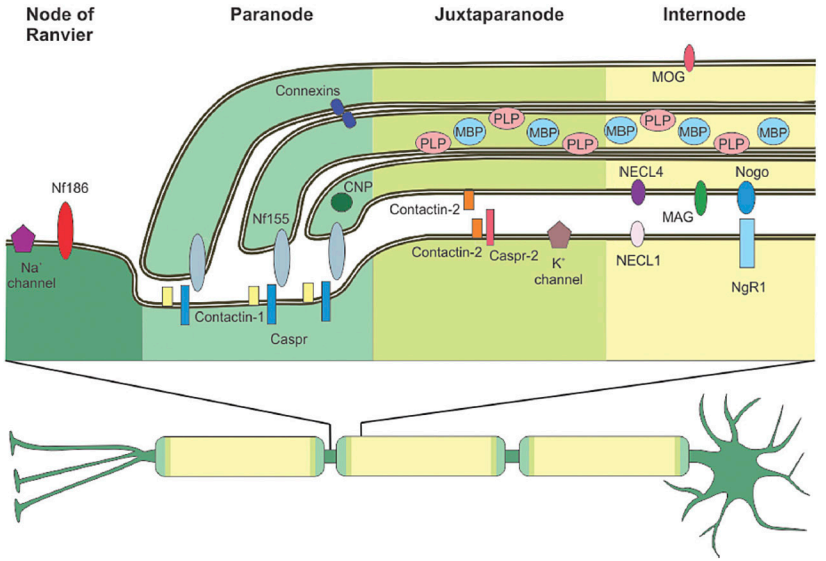


Figure 1: The lower part of the figure depicts a neuron (the stellate cell on the right) with its extension (axon), which is enwrapped by bands of myelin interspaced with non-myelinated nodes of Ranvier. The nodes are essential for fast pulse conduction, which jumps from one node to the next. The upper part depicts an enlarged segment, showing the layers of myelin in which a selection of essential proteins is indicated. Note that only myelin oligodendrocyte glycoprotein (MOG) is exposed on the myelin surface. (figure: Mayer and Meinl, Ther. Adv. Neurol. Dis. 2012)

Our research approach

Intensive research over the past decades has delivered a number of therapeutic agents with reasonable efficacy in RRMS. However, for progressive MS there is no effective treatment available. The main reason for this situation, which is rather disappointing taken the substantial research efforts, is our still very limited understanding of the factors that underlie the induction and perpetuation of the disease. In our preclinical research we integrate exploratory research into

disease mechanisms and the causal relation between infection and MS with applied research into how new therapies work to maximize relevance for the patient (Figure 2).

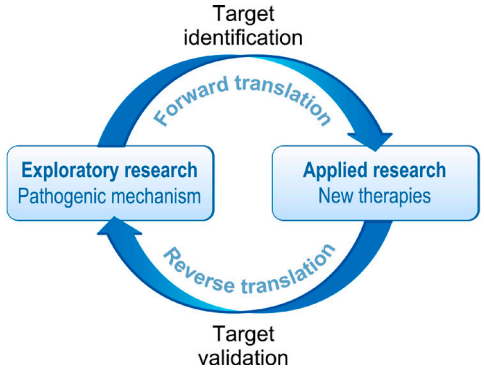


Figure 2: integration of exploratory and applied research in our preclinical research

The role of animal models in MS research

Despite the many uncertainties, relative consensus exists on the concept that MS is caused by the interaction of genetic and environmental factors, which together induce the pathogenic immune reactions. This concept is the basis of most MS research. For genetic and immunological studies in patients blood and, albeit more difficult to collect, the cerebrospinal fluid that surrounds the CNS, are used. However, the CNS itself is such a vulnerable organ that tissue samples for research cannot be collected without the risk of irreversible damage. These limitations have created a need for valid animal models in which clinical and pathological aspects of MS can be investigated and new therapies can be developed.

The elected animal model in MS research is experimental autoimmune encephalomyelitis (EAE), which has been established in mice, rats, guinea pigs and monkeys, which resembles the situation in humans at best.

The non-human primate EAE model

The impressive biotechnological development in the past decades has created the possibility to generate biological molecules of high quality and sufficient quantity for the treatment of patients. With these "biological bullets" cells and molecules that induce and/or perpetuate the disease can be functionally or physically eliminated in a highly specific manner that spares tissues and organs not involved in the disease.

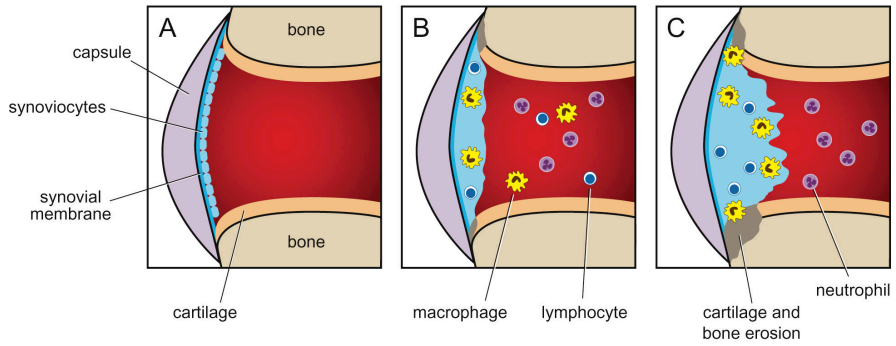
Over the years we have developed well-validated EAE models in the small-bodied common marmoset (*Callithrix jacchus*), which recapitulate essential clinical and pathological aspects of relapsing-remitting and of progressive MS.

Major achievements

- Implementation of magnetic resonance imaging (MRI) techniques with which development of MS-like lesions in the brain could be longitudinally monitored. The possibility to perform brain MRI has been an essential tool for assessing effects of a new treatment on existing disease.
- The discovery that the initiation and progression of EAE in marmosets are driven by distinct pathogenic mechanisms. The specificity, MHC restriction and mode of action of the T cells that operate in EAE initiation and progression has been resolved.
- The discovery that virus-infected B cells have a key pathogenic role. Development of a highly refined EAE model induced with a synthetic peptide in oil.

Rheumatoid Arthritis

With a prevalence of 1%, rheumatoid arthritis (RA) is one of the most common immune-mediated inflammatory diseases in the human population. RA primarily affects the joints of all extremities and is pathologically characterized by severe inflammation and progressive destruction of cartilage and subchondral bone.



Severe inflammation of a healthy joint (A); thickening of the synovial membrane and the involvement of immune cells (B) and subsequent destruction of cartilage and subchondral bone.

In the preclinical research of RA, aiming at a better understanding of disease mechanisms and development of safe and effective treatments, animal models have an important role. It is of critical importance that the animal model used is sensitive to the pharmacological action of the tested drug and that the tissue distribution and pharmacological properties of the molecules targeted by the treatment are comparable to those observed in patients. The BPRC has developed 2 NHP models of inflammatory arthritis: A collagen induced arthritis (CIA) model in the rhesus monkey and in the common marmoset.

CIA in the rhesus monkey

With the use of genetically susceptible rhesus monkeys we have developed a NHP model of inflammatory arthritis. A one-time injection with collagen type II (CII) in a strong immune-stimulating formulation results in a mild to severe swelling of the peripheral joints. Onset of clinical arthritis occurs 3 weeks after the immunization. Frequent physical examination and blood analysis provides a detailed analysis of the inflammatory response developing in these animals. The available biomarkers provide quantitative information on: **1**) the clinical status (e.g. disease score, soft tissue swelling of the joints, bodyweight and body temperature), **2**) severity of inflammation (e.g. serum C-reactive protein, IL-6, platelets, neutrophils, haematocrit), **3**) immune status (serum autoantibody levels, T-cell reactivities and immune phenotyping) **4**) joint pathology (serum alkaline phosphatase, urinary excretion rate of collagen crosslinks and end-stage histopathology). The size of the animal allows for frequent blood-sampling for in depth analysis of immune parameters. The rhesus monkey CIA model has been validated with clinically relevant therapeutic agents, namely a monoclonal antibody against the human IL-6 receptor (Tocilizumab) and a fusion protein CTLA-4Ig (Abatacept).

CIA in the common marmoset

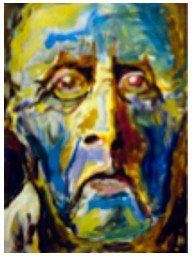
Recently we developed a new CIA model in a New World primate, the common marmoset. Induction of visible arthritis required immunization with CII in a strong adjuvant followed by several immunizations with CII in oil emulsion. This model displays 2 major disease patterns. In about 50% of the animals clinical signs develop approximately 4 weeks after immunization. The other half of the animals develops a more chronic disease. Both helper T and cytotoxic T cells as well as antibody responses (IgM & IgG) against CII were implicated in

the development of the disease. At the level of histopathology, tissue analysis showed less destruction of bone and cartilage compared to the rhesus monkey CIA model. We also observed unique extra-articular manifestations of inflammation near the bone outside the joint and subcutaneous tissues in acute responders to disease induction. Because of the small size of an adult monkey (± 400 grams) the model is ideal for the efficacy evaluation of a new drug at early stage development, when only limited amounts of drug are available. A unique feature of the common marmoset is that they are born as non-identical twins or triplets with chimeric bone marrows due to fusion of the placental bloodstreams. This creates the unique situation that the twin sibling of a monkey in an experimental group can be used as the optimal control. The model still awaits validation with a clinically relevant therapeutic.

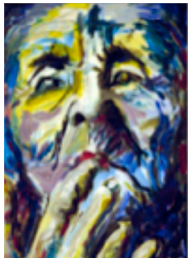
Both models provide an important platform for the evaluation of human specific therapeutics. These models can also identify the selective impact that a drug might have on processes that show comparable expression and regulation in humans and NHP, but are lacking in rodent models because of the evolutionary distance. The models also provide the opportunity to perform exploratory research into comorbidities of RA, including anemia and atherosclerosis.
Recent comparison of the MHC genomic regions of the common marmoset and rhesus monkey revealed presence of MHC alleles that contain the shared epitope. This is a motif in the peptide binding groove of the DRB1 molecule that is linked to more severe arthritis, the presence of diagnostic antibodies against citrullinated proteins and smoking. Especially, a large proportion of the common marmoset express MHC molecules with this particular motif. This creates a specific niche exploring the impact of this genetic trait in the disease course of arthritis.

Alzheimer & Parkinson's disease

The increasing prevalence of neurodegenerative diseases is a growing concern for ageing societies. A highly prevalent age-associated neurodegenerative disorder is Alzheimer's disease. At this moment there are about 35 million patients worldwide and every 2 seconds, this disease affects a new person. Another major neurodegenerative disorder is Parkinson's disease. Nowadays about five million patients have been diagnosed with Parkinson's disease worldwide.



Parkinson's disease (PD) is a motor dysfunction disorder caused by specific progressive degeneration of dopamine containing nerve cells (neurons) in the *substantia nigra*, a small nucleus in the brain that is involved in control of movements. This results in a wide range of motor symptoms, such as slowness of movements, tremor, rigidity and/or postural instability. At the time the patient is diagnosed at least 50-60% of the dopamine producing cells in the brain are already lost.



Alzheimer's disease (AD) is a brain disease that slowly affects memory, and the ability to carry out simple tasks of daily living. About 50-80% of all cases of dementia are caused by AD. As cells die over time, affected brain regions begin to shrink. This degeneration seems to be woven by aggregation of abnormally-folded proteins, such as amyloid plaques, that are causally linked to cellular stress and inflammation in the brain.

Treatment of neurodegenerative diseases

AD as well as PD are both irreversible, progressive brain diseases for which no cure exists. Current medications suppress disease symptoms, but do not affect the progressive degeneration of neurons in the brain. Hence, there is a pressing need to tap into treatments aiming at protecting the brain against neurodegeneration. Since the actual cause of PD and AD is still unknown, it is difficult to develop disease-modifying treatments to stop or slow down the disease progression at an early stage.

- In AD, protein misfolding, after it has originated locally, spreads through the brain as if it is a "contagious" or prion-like disease. An inflammatory response may well be responsible for this progressive spread throughout the brain. These aggregations of proteins hinder communication in the brain that finally results in disconnections, brain cell death, and memory loss. Treatment of AD is still focussed on mitigation of cognitive decline for as long as possible. As the progression continues this battle will be lost and memories will fade away.

- The PD patient encounters the same problem as treatment relies on dopamine replacement therapies that are focussed on improving motor function. Besides the fact that progressive degeneration continues, these therapies induce severe side effects, such as dyskinesia (abnormal involuntary movements) caused by levodopa medication, which is one of the most important concerns of the patient. Strategies to limit these side effects are of high priority in order to improve the quality of life of the patient. There is a strong need to develop treatments to restore normal function after levodopa treatment in PD patients permitting long-term

medication. The Michael J. Fox Foundation has awarded a study to evaluate a candidate compound, HE3286 (Triolex), to prevent these undesired side effects. HE3286 blocks the activation of the transcription factor pathway in the neuron and blocks the NMDA receptor on the surface of the neuron, which is involved in the dyskinesia and in the calcium homeostasis, a feature in neurodegeneration in general (Figure 1).

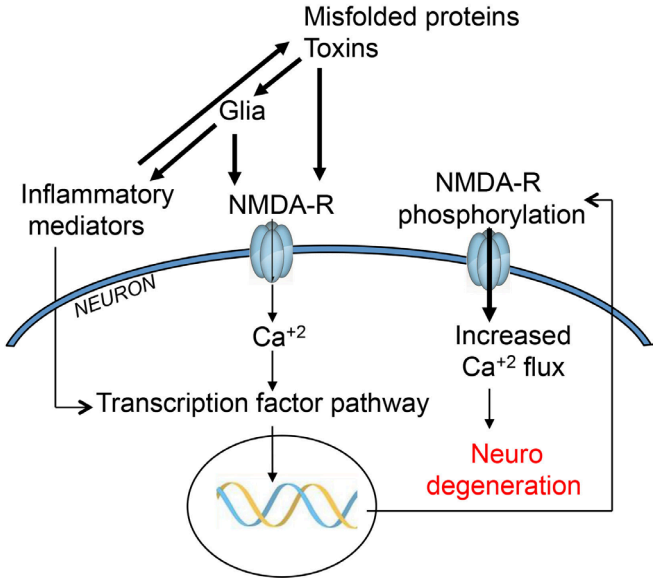


Figure 1: schematic overview of the interaction between protein misfolding or toxins with supporting cells, named glia, and with neurons in the brain and the mechanism of action in these neurons. Over-activation and phosphorylation of the NMDA receptor by damaging factors (a.o. toxins, activated glia cells) causes a disturbed calcium homeostasis leading to cell death as seen in PD and AD. Inflammatory mediators increase this process. The NMDA receptor and activated transcription factors are also responsible for levodopa induced dyskinetic side effects in PD.

The relevance of monkeys in the research

Animal models offer an opportunity to link low-level neurodegeneration to disease manifestation and a possibility to investigate strategies for intervention therapy. While physiological and pharmacological questions can often be studied in rodent models, issues concerning complex behavior and cognition can be addressed more accurately in non-human primates (NHP), which optimize the extrapolation of the results to human.

The common marmoset (Callithrix jacchus) is an established model in neuroscience:

- Marmosets develop, like humans, natural protein plaque progression during aging and cognitive decline, which might reflect their genetic, physiological and behavioral proximity to humans (Figure 2). Injections of beta-amyloid protein in the brain of marmosets in combination with an activated immune system might trigger the spread of plaque formation in the brain as seen in AD. This model validation is currently being performed at BPRC.

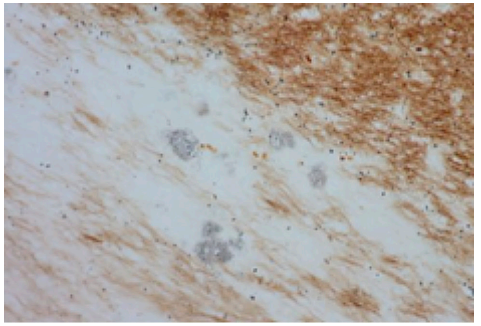


Figure 2: Example of spontaneously developed diffuse amyloid plaques (black spots) in brain tissue of a 7-year old female marmoset monkey with wasting syndrome that had been exposed to inflammation during the last 6-months of her life. Stained with a Campbell-Switzer staining.

Alzheimer & Parkinson's disease

- A well-validated approach for the induction of PD is exposure to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP-treated marmosets show optimal face validity with a wide range of parkinsonian behaviors, including the levodopa-induced dyskinesia. We have implemented a progressive-chronic PD model in the marmoset, which is based on long-term chronic exposure of low doses of MPTP, which selectively damage dopamine neurons that play a role in motor function. This approach best reflects idiopathic PD and is comparable with the accidental disease induction in drug users exposed to MPTP.

Sensitive non-invasive read-out systems for motor function, cognition but also for sleep disturbances and other behavioral symptoms with an open eye towards human validity are used to identify the symptomatic dynamics during disease development and the effects of candidate treatments. Post-mortem proteomics and transcriptomics of key brain structures are used to identify changes in protein and gene expression to find new targets for neuroprotection.

Malaria

Malaria-the disease and research

Malaria is the biggest parasitic killer in the world. Malaria is caused by *Plasmodium* parasites which are transmitted from man-to-man by mosquito. In humans, the parasite first infects the liver, where it multiplies several thousand-fold in a week or so, without giving any disease symptoms. Then, the parasites escape from the liver and infect red blood cells. The ten-fold cyclical multiplication in only 48 hours gives rise to malaria symptoms.

There are two major human malaria parasites, *P. falciparum* and *P. vivax*. *P. vivax* is very difficult to study as, unlike *P. falciparum*, the parasite cannot be cultured *in vitro*. Thus, the only sources of parasites are patients, or certain experimentally infected South American monkeys that are very scarce.

Malaria research in the Department is resting on three pillars: drug development, vaccine development, and malaria parasite biology. The biology, including parasite-host interactions, forms a red thread through all our research as we believe that if we understand the parasite better, we can make better working vaccines and drugs for the combat of malaria.

Malaria drug development model

For our drug development programme we focus on dormant liver stage parasites of *P. vivax*, a unique stage that is only present in *P. vivax* and a few closely related primate malaria species. Due to the difficulties described above, we are using a relative of *P. vivax*, the primate malaria *P. cynomolgi*. Using infected rhesus monkeys as a source of parasites, we have developed an *in vitro* drug assay for the dormant liver stage parasites that we use for the screening of potential new medicines. This is important, as in humans these stages form a hidden parasite reservoir that can give rise to new blood stage malaria at any time.

New medicines are necessary as currently only a single medicine exists and parasites become resistant to this medicine. In 2013, we have optimized an active compound which will be further tested *in vivo* in the *P. cynomolgi*-rhesus monkey model for activity. Furthermore, to study the biology of the dormant liver stages, we have been able to purify sufficient dormant parasites from *in vitro* culture to allow extraction of genetic material for analysis. The results will tell us which building blocks are present in the dormant parasites, which may lead to new targets for medicine development.

(Pre)clinical malaria vaccine development

In our vaccine programme we focus on the blood stages of *P. falciparum*, for which we have developed a good vaccine candidate, PfAMA1. In the past we had developed *in vitro* assays to be able to measure whether immune responses against PfAMA1 can inhibit parasite growth, and used rhesus monkeys to evaluate safety and immune responses of different PfAMA1 formulations, suitable for usage in humans. The immune responses were measured using *in vitro* assays; as rhesus monkeys cannot be infected with *P. falciparum*, protective efficacy of the vaccination cannot be measured. Based on all these studies, we have selected suitable formulations in 2013 and made preparations for clinical evaluation of the PfAMA1 vaccine candidate in 2014.

Biological studies in non-human primates

We focus our biological studies on non-human primate malarias that are close relatives of *P. vivax* (*P. cynomolgi* and *P. knowlesi*) because **1**. the parasites are closely related to human parasites, and **2**. the host, the rhesus monkey, is closely related to humans. Thus, outcomes of studies can be easier extrapolated to the human situation. In 2013 we started a study where we vaccinate rhesus monkeys with *P. knowlesi*-infected mosquito bites in the presence of a strong anti-blood stage malaria medicine, which does not interfere with liver stage development but prevents blood stage malaria. This is a known procedure to experimentally protect people from infection, but we do not understand exactly how this works. The purpose of this experiment is to find mechanisms of protection by detailed analyses of protective responses in general, but uniquely also locally in the liver, spleen and lymph nodes, important immunological organs, that cannot be obtained in human studies. Results will follow in 2014.

Female Anopheles mosquito, carrying malaria parasites in the saliva, used for vaccinating rhesus monkeys (Picture from www.sciencebuzz.org)

Tuberculosis

A Global Health Challenge

Worldwide, tuberculosis (TB) kills one human being every 20 seconds. The infection is caused by inhalation of *Mycobacterium tuberculosis* (*M.tb*) and relatively difficult to treat with antibiotics. To fight TB at a global scale, vaccination – that is prior harnessing of immune defense to prevent pathogenic *M.tb* from doing harm - will be most effective. The current vaccine, live *Mycobacterium bovis* BCG, however, often fails in different populations. Since the mechanisms of protective immunity are poorly understood, it remains a formidable challenge to improve TB vaccine therapy beyond the level of BCG.

Why Using Non-Human Primates?

More research is required and clinical studies are informative, yet suffer greatly from the extended incubation time that can occur upon (latent) infection with *M.tb*. Experimental infection of humans to control kinetics, however, is considered as undesirable. Thus, model systems are indispensible and, so far, only animal models harbour the immuno-physiological complexity that underlies disease development (pathogenesis) and protective immunity.

We use non-human primates (NHP) and macaques specifically, which – in contrast to most if not all other TB models - are closely related to humans, are naturally susceptible to *M.tb*, and present the best recapitulation of the human disease. NHP are used for testing new vaccination strategies and for investigating mechanisms of protection.

Our previous NHP studies have suggested that different cohorts of macaques respond differently to infection with *M.tb* and vaccination with *M.bovis* BCG, resembling the situation in clinical TB. To exclude any possible variation due to disparity in mycobacterial challenge and/or experimental conditions, we planned a head-to-head comparison of two distinct breeding populations of rhesus macaques in a BCG vaccination and *M.tb* infection study. By investigating the differential macaque responses we hope to learn of mechanisms that determine TB susceptibility and protective efficacy or failure of BCG vaccination as it occurs in man.

Our Latest Results

Our head-to-head comparative study confirmed that animals from different breeding origin differ in susceptibility to *M.tb* infection and TB disease. Rhesus macaques of Indian breeding origin presented with significantly more pathology as compared to the Chinese-type rhesus macaques. Accordingly, bacterial burden and various clinical measures were differentially affected between the two cohorts.

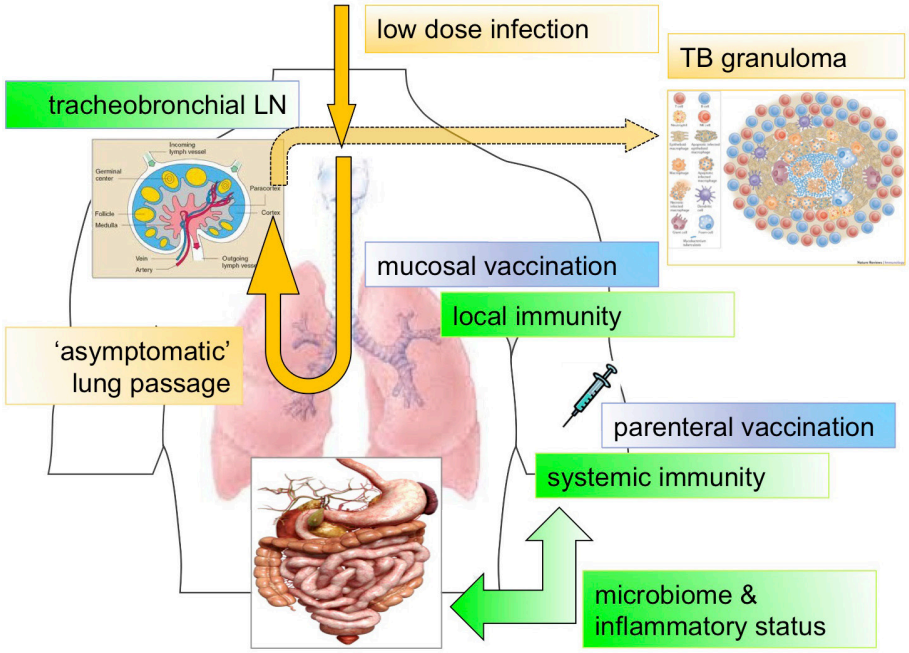
We also found that only one of two groups showed reduction of disease as a result of prior BCG vaccination (although the potential of BCG to protect, does not strictly correlate with breeding origin). Remarkably, upon vaccination, T cell immune responses that are typically addressed by vaccination, were not found to be different between the two rhesus monkey groups. On the other hand, changes in myeloid phagocytic cells, which play an important role in orchestrating T cell immunity, were distinctive already one week after vaccination. Similarly, phagocytic myeloid cells showed differential responses between cohorts early after *M.tb* infection. Recent clinical studies suggest that the response status of such myeloid cells, rather than T cells, predicts the risk of developing severe TB disease. Our rhesus macaque study results would extend upon those observations.

Additionally, we investigated local vaccination by unconventional pulmonary (lung) administration of BCG. Most notably, in the rhesus cohort where standard intradermal (skin) BCG treatment had no effect, vaccination via the

lung still did reduce lung pathology significantly. This underpins the relevance of addressing the lung compartment by local vaccination to reduce pulmonary TB, and provides for a treatment strategy that should be pursued in our future research.

This study has provided a large set of data and biosamples that need to be explored further and from which we may expect to learn about the mechanisms that determine TB disease suceptibility and the success (or failure) of BCG vaccination. Our data immediately impact on refined use of NHP macaques, and on the design of future studies to unravel the mechanisms of local immunity. Ultimately, these findings and the ongoing analyses contribute to our knowledge-base and should be instructive towards developing better therapies for human TB in the near future.

Tuberculosis infection and immune compartment interactions



Recent results from our NHP studies and reports in literature do underpin the notion that the lymphatic system is critically involved and also at early stages of TB infection. Early changes in the systemic myeloid immune compartment (or the inflammatory status) seem to correlate with intradermal (parenteral) TB vaccine effects. At the same time we have shown that targeting local immunity by vaccination can significantly improve towards TB lung disease. These interactions of local and systemic immune modulation will shape our future research strategies.

Viruses & Vaccines

Viruses are very small particles made of proteins and lipids encapsulating their genetic material (DNA or RNA). Viruses cause familiar infectious diseases such as cold, flu, diarrhea, and warts, and can also cause severe illnesses such as HIV/AIDS, smallpox and hemorrhagic fevers. Viruses behave like hijackers as they invade cells and use them for multiplication and production of new virus particles. This can eventually kill infected cells and cause disease. Viral infections are hard to treat. Antibiotics work on bacteria, but do not act on viral infections. Viruses that persist inside the body's cells are "protected" from most medicines. Vaccines can protect against getting viral infections and/or diseases. Therefore vaccination is the best approach for the treatment and prevention of viral infectious diseases. At the department of Virology we focus on developing and testing potential vaccines against several viral infections. In our research animal infection models are used for studying the efficacy of potential vaccine candidates. We have established infection models for HIV/AIDS, Influenza virus, and West Nile virus in non-human primates (NHP). The latter two models were established in 2013. Besides testing the efficacy of various potential vaccine candidates, we also investigate how vaccines work in the combat of viral infections in order to improve vaccine candidates.

Use of non-human primates in virus research

Animals (including humans) have dedicated cells and tissues to deal with the threat of infection. Infection usually leads to the induction of an immune response. Some responses occur immediately so that an infecting agent can be quickly contained. Other responses are slower but are more focused to the infecting agent. Infection leads to disease. The human immune system has two levels of immunity: specific and non-specific immunity. Through non-specific immunity, also called innate immunity, the human body protects itself against foreign material that is perceived to be harmful. These first lines of defense include outer barriers like the skin and mucous membranes. When pathogens (like bacteria and viruses) breach the outer barriers, for example through a cut in the skin or when inhaled into the lungs, they can cause serious harm. In contrast to innate immunity, specific immunity, also called adaptive, allows for a targeted response against a specific pathogen that can last for years because memory immune cells are induced.

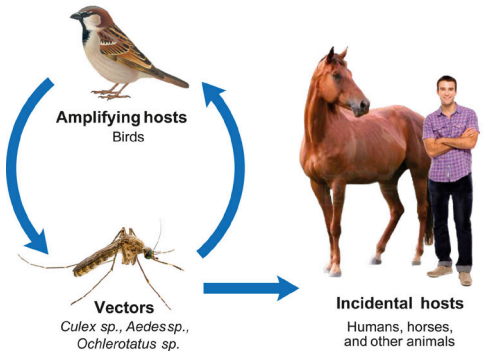
Vaccination stimulates a specific immune response that will generate the expansion of immune cells (memory B and T cells) specific to a certain pathogen. The memory cells persist in the body and can lead to a quick and effective response should the body encounter the pathogen again. Part of these processes can be studied in laboratory dishes (*in vitro*), but the complex interactions between many of these immune cells can only be studied in a living animal (*in vivo*). Because we develop vaccines against human viral diseases, it is important to use animal models that 1) display susceptibility to infection with these human viruses, 2) mimic disease progression in humans, and 3) have immune systems that are comparable to humans to study the mechanism(s) of protection. Only with adequate animal models we can study the efficacy of potential vaccine candidates for human purpose. For instance human influenza virus and West Nile virus can both infect mice. However, when infected with these viruses the animals die within a couple of days. This does not mirror the human infection where the virus is normally cleared from the body within 2-3 weeks after an initial disease. Another example is the HIV/AIDS model. This virus cannot infect other mammals than non-human primates. Although humanized mice have recently been developed as a model to study HIV infection, the immune system and responses in non-human primates most closely mimics that of humans and the non-human primate model is therefore essential for evaluation of human vaccine candidates.

Results obtained in 2013

In 2013 we have established different influenza virus and West Nile virus infection models. These models are now being used to study vaccine candidates for their immunogenicity (does the vaccine stimulate high immune responses?) and efficacy (are the induced responses potent enough to protect?) against viral infections and/or disease progression. Additionally, these models can also be used for the study of other intervention strategies for instance anti-viral monoclonal antibodies and anti-microbial compounds.

Emerging viruses

Emerging virus diseases are a major threat to human and veterinary public health. Viruses like Dengue virus, Chikungunya virus, and West Nile virus have in recent years caused epidemics in Europe. In nature, West Nile virus (WNV) cycles between mosquitos and birds, but is incidentally transmitted to humans and other mammals, like horses, by infected mosquitoes (see figure). In most humans, WNV infection goes unnoticed or leads to a flu-like illness, but in 1% of human WNV infections it leads to a serious, sometimes fatal disease. In the recent years we have set up a WNV infection model in rhesus macaques and common marmosets that can be used for vaccine testing or evaluation of antiviral therapies. In the past year we successfully evaluated two WNV vaccines in rhesus macaques. Both vaccines, both based on the WNV envelope protein, fully protected macaques against virus in the blood, and drastically reduced the number of infected cells in various tissues in the vaccinated animals. Additionally, we have seized this opportunity to develop serological assays for the detection of WNV and Dengue virus antibodies in sera of non-human primates. These assays are now part of the Primate Viral Diagnostics service that we offer to primate-keeping institutes around the world.



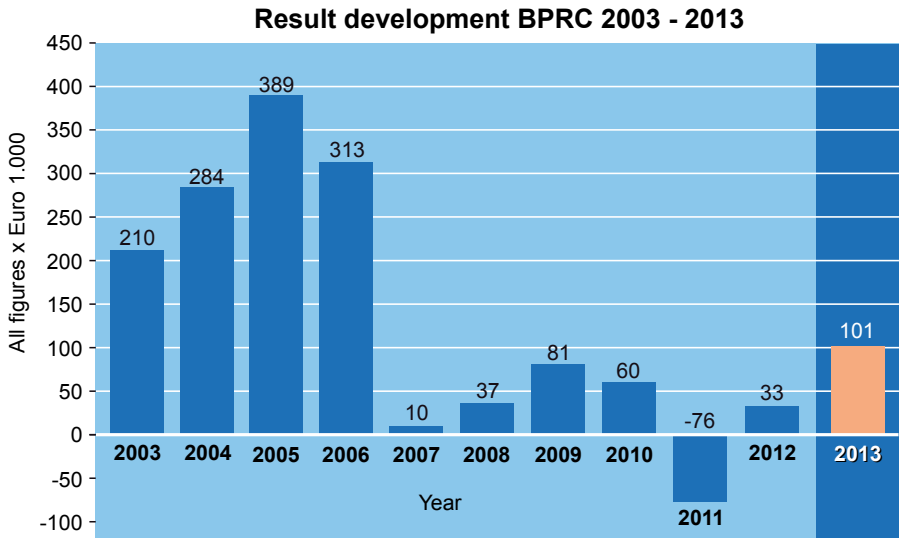
Influenza viruses

Timeline of human influenza pandemics including human cases of avian and swine influenza viruses.

During seasonal epidemics, influenza viruses cause approximately 250,000-500,000 deaths annually. Young, elderly and immune-compromised people are those mainly at risk for developing severe disease. Recently, influenza A viruses of swine origin have caused the first pandemic of the 21st century. Animal models play an important role in studying the pathogenesis of influenza virus infections. Non-human primates are closely related to humans and show immunological and physiological resemblances/similarities that make them a highly relevant model in pre-clinical safety, immunogenicity and efficacy evaluation of vaccines and therapies. In 2013 we have set up an influenza virus infection model with a swine origin pandemic influenza A virus and compared susceptibility to infection in three different non-human primate species. The information obtained allows us to select the most optimal model for evaluation of novel "universal" vaccine candidates, which are currently being developed by the EU-funded EDUFLUVAC consortium (www.euvaccine.eu/portfolio/project-index/edufluvac). This consortium is working on the development of a vaccine that will protect against many different influenza virus variants, including seasonal variants that emerge each year as well as newly emerging viruses. With such a vaccine the yearly vaccination would no longer be necessary and the threat of future influenza epidemics would be reduced.

Finance

The BPRC financial annual report has been audited and approved by our financial accountant.



PROFIT AND LOSS ACCOUNT

	2013 (K€)	2012 (K€)
Turnover projects (extern)	5.781	5.711
Turnover projects (subsidy)	6.418	6.312
Total turnover projects	12.199	12.023
Other excluding interest	392	1.261
	392	1.261
Total turnover	12.591	13.284
External direct project costs	770	867
Staff costs	7.537	7.775
Depreciation	508	625
Other operating charges	3.910	4.087
Total operating costs	12.725	13.354
Profit/loss on ordinary activities	134-	70-
Interest	235	103
Profit for the financial year	101	33
Tax	-	-
Profit for the financial year after tax	101	33

EFFECTIVE PERSONNEL

	2013		2012	
Service Departments	15.1	14%	15.3	13%
Animal Sience Department	43.9	40%	46.3	40%
Research	49.9	46%	54.2	47%
Total	108.9	100%	115.8	100%

BALANCE SHEET 31 DECEMBER

	2013 (K€)		2012 (K€)	
ASSETS				
FIXED ASSETS				
Buildings and structures	41.224		42.886	
Tangible fixed assets	1.617		1.761	
		42.841		44.647
CURRENT ASSETS				
STOCKS		52		38
DEBTORS DUE WITHIN ONE YEAR				
Work in progress	1.614		1.384	
Receivables from contracts	386		1.544	
Receivables tax	255		463	
Other receivables	60		258	
		2.315		3.649
Cash at bank and in hand		18.979		15.902
Total assets		64.187		64.236

	2013 (K€)		2012 (K€)	
LIABILITIES				
EQUITY				
Equity	2.317		2.284	
Revaluation reserve buildings	7.642		8.252	
Result current year	101		33	
		10.060		10.569
PROVISIONS				
Primates	104		205	
Deferred tax liabilities	2.547		2.750	
(Flexibel) retirement	-		-	
Repairs buidings	6.009		5.189	
		8.660		8.144
LONG TERM DEBTS				
Bank	26.803		27.569	
Received in advance on asstes	11.567		11.688	
		38.370		39.257
SHORT TERM DEBTS				
Received in advance on projects	3.153		2.193	
Received in advance on assets	130		210	
Received in advance subsidy	160		480	
Accounts Payable (TAX)	476		524	
(Flexibel) retirement	-		-	
Accounts Payable	473		428	
Commitment Bank	766		729	
Other liabilities	1.939		1.702	
		7.097		6.266
Total liabilities		64.187		64.236