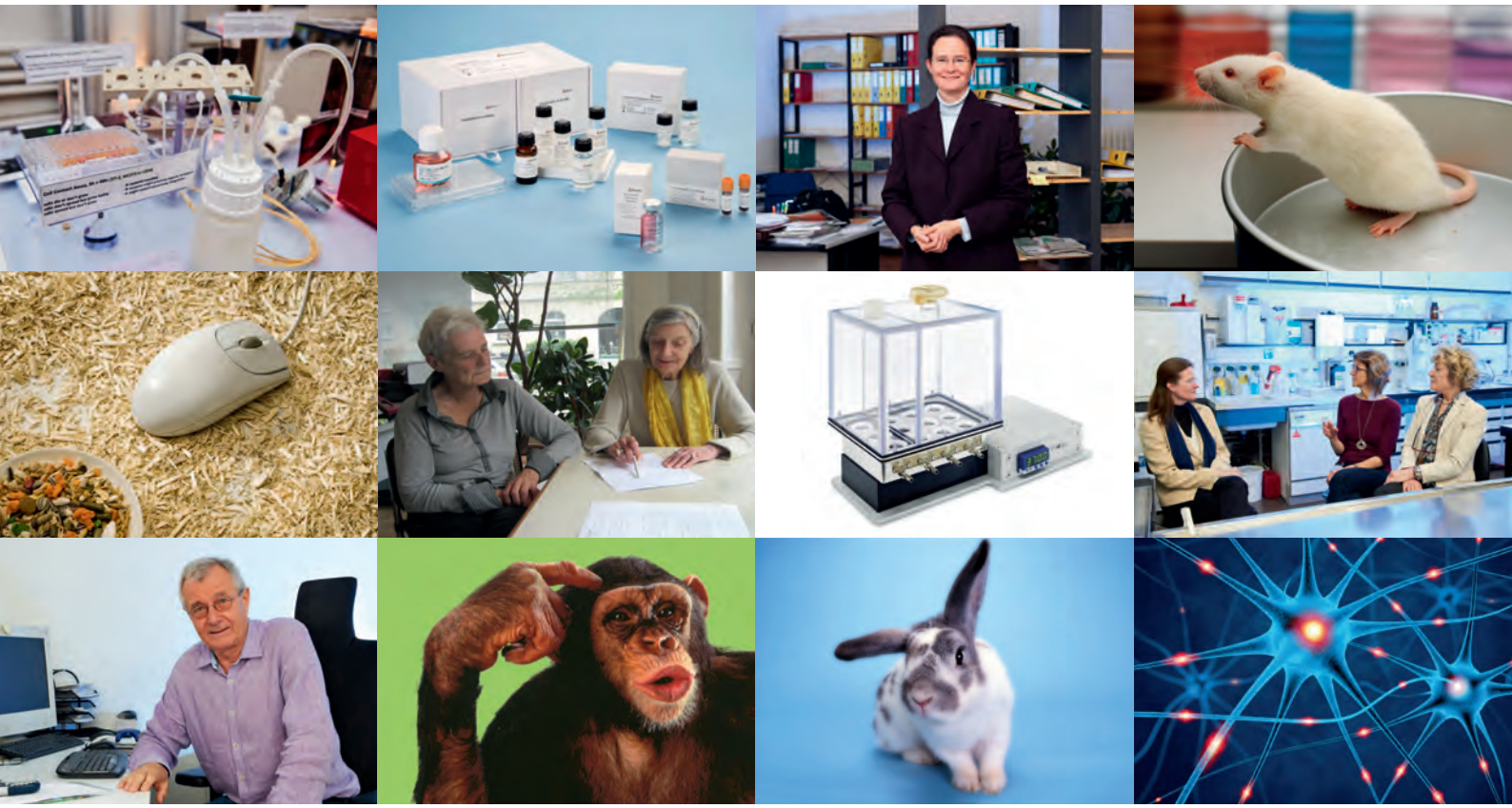
AnimalfreeResearch

replacing animal experiments



Quality Assessment Final Report

to the Animalfree Research Foundation
Evaluation of Funding Activities 1976 – 2016

Author: Dipl. biol. Monica Gaiffi

Quality Assessment

Final Report

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This study was conducted on behalf of the Animalfree Research foundation (AfR). The contracting authority did not influence the outcome of the study in any way. The responsibility for the results lies solely with the author. The standpoints expressed in this study are those of the author and do not necessarily reflect those of AfR.

Monica Gaiffi established contact with our foundation with the explicit wish to contribute to our work. She was tasked with writing this report since she has a solid scientific background and a deep interest in animal welfare and the 3R concept. At the same time, she was independent and had never worked for Animalfree Research before.

This document is based in part on a former unpublished report written by Dr. Stefanie Schindler on behalf of another foundation. If contents were adopted, they were strictly limited to general considerations and definitions and did not influence the project evaluation.

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1. Introduction

1.1 Aims and scope of the study

In 2016, the Animalfree Research (AfR) foundation celebrated its 40th anniversary. From 1976-2007, the Foundation's name was "Fonds für Versuchstierfreie Forschung" (FFVFF). For 40 years, this foundation has been helping scientists to fund their research with the aim to help develop new methods based on the 3R principles: reduce, replace and refine the use of animals in research (see chapter 2.1.1). To receive funding, a scientist needs to show that his/her project aims to contribute to these goals, e.g. by helping to replace in vivo models with in vitro models which do not use, or hardly use, any animal tissue, reducing the number of animals in a commonly used type of experiment or by enhancing the validation or acceptance of methods that have the potential to contribute to the objectives of the 3Rs principles. After funds are granted, the scientists can profit not only from the money given by AfR, but also tap into the foundation's scientific expertise and network of researchers in a wide range of scientific fields.

The aim of this study was to follow-up on projects which have been either partly or fully financed by AfR/FFVFF over the last 40 years. To achieve this, the author of this report looked into every project individually. By researching the internet, reviewing old documents and by contacting former project leaders or project members, she assessed the impact that each project had on future research up to the present day - with the main focus on the outcome of the project in supporting the replacement, reduction and refinement of the use of animals in research.

1.2 Background: the 3R concept

To understand the importance of the 3Rs and the necessity to fund 3R-related projects, animal numbers, severity degrees and other 3R related developments in Switzerland in the past years have to be taken into account.

In 2015, 682'333 animals were used in Switzerland for research purposes. These are huge numbers. They nevertheless have to be seen in a certain context, which is the development of overall laboratory animal numbers since 1983 – the first year the Federal Veterinary Office (FVO) issued an official statistic.

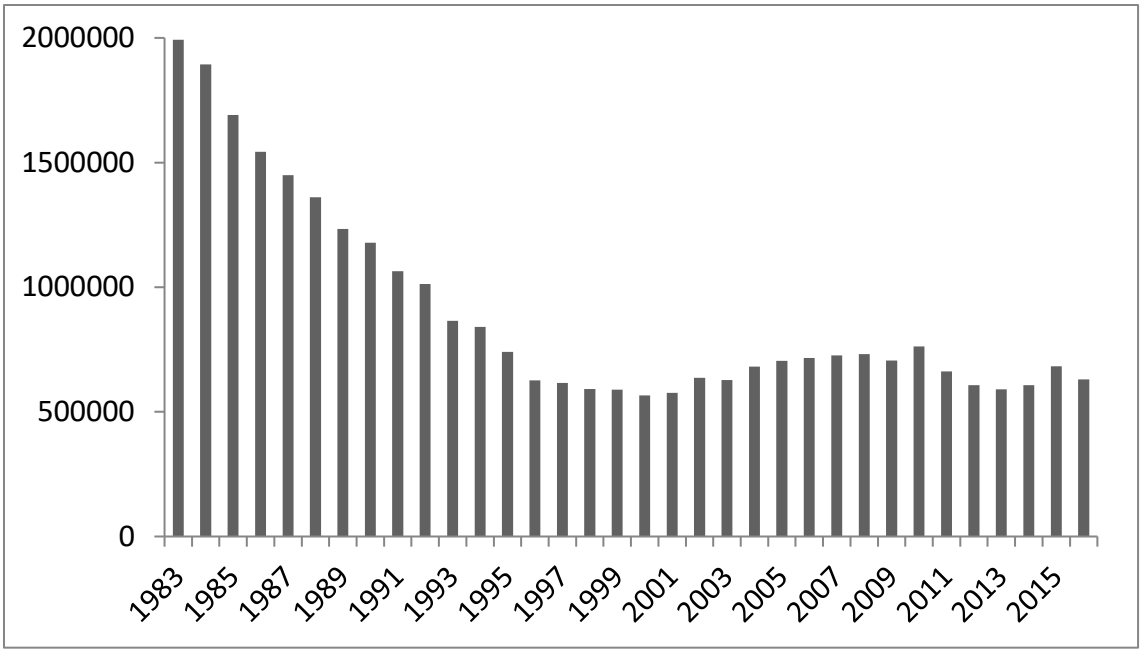

Fig. 1: Total experimental animal numbers in Switzerland, 1983 – 2016 [1]

At time of the founding of the FFVFF in 1976, the numbers of animals used in experiments were not yet documented, but were most likely at a similarly high level as in 1983 when documentation started. With an extreme high of almost 2 million animals, action was necessary and successful – we were able to witness significant reduction in animals used in experiments over the years (Fig.1).

Alternative methods, (Replacement, Reduction, Refinement = the 3Rs) were first described by the British scientists William Moy Stratten Russell and Rex Leonard Burch. Upon appointment by the Universities Federation for Animal Welfare (UFAW), they conducted a systematic survey among scientists on the progress of humane research. For this, R.L. Burch travelled through Britain and interviewed researchers, with W.M.S. Russel doing the analysis and writing the report [2]. The result was a strictly scientific concept, which became known as the 3R principles/the 3R-concept or simply the 3Rs. Since then (with a certain latency) the 3R concept has been accepted universally. It has also been explicitly endorsed nowadays, since the 3Rs are gaining a foothold in nearly every area: industry, academia, funding schemes, publication possibility, etc.

The declaration of Bologna, adopted at the 3rd World Congress on Alternatives and Animal Use in the Life Sciences (1999) defines the 3Rs as follows:

Replacement: Methods which permit a given purpose to be achieved without conducting experiments or other scientific procedures on animals. Since replacement may well involve the use of animal cells or tissues, and therefore the painless killing and use of the animals, there is a further distinction between “absolute” and “relative” replacement, the former indicating that no animal was affected at any time in the proceedings.

Reduction: Methods for obtaining comparable levels of information from the use of fewer animals in scientific procedures or for obtaining more information.

Refinement: Methods which alleviate or minimize potential pain, suffering and distress, and which enhance animal well-being.

1.3 The „Fonds für Versuchstierfreie Forschung“ (FFVFF)

The completely self-supporting foundation FFVFF was founded in 1976 by Susi Goll, Irène Hagmann, Max Keller and Max Neidhard and was originally based in Zürich, Switzerland. The foundation’s aim was to raise money to support scientific projects which, in the long term, were promising for completely replacing the need to use animals in particular types of studies.

1.4 The Animalfree Research foundation (AfR)

The FFVFF was renamed into Animalfree Research in 2007, taking into account the increasing importance of international collaborations. There was no change of the guiding principle, which is until today: “We Replace Animal Experiments”, accomplishing this by supporting research projects with a clear 3R objective. The main focus lied - and still lies - clearly on projects aiming at replacement or reduction (rather than refinement) of animal experiments.

Contrary to conventional research, which puts the scientific and economic interest first, AfR only supports research which is inspired by an animal-welfare point of view. This is often driven by scientists themselves, who may have observed problems or ethical concerns when performing their work in the lab.

For AfR it is not only important to support researchers but also to make sure that the results of their work get published. As a consequence, every supported researcher is obliged to sign a contract with AfR, making sure that the results will be published in a scientific journal and therefore communicated to other scientists around the world. At the same time, AfR uses its different channels to distribute the results to the general public.

1.4.1 Structure and funding of AfR

The AfR consists of a steering committee which is led by a president (since 1/2016 two Co-Presidents), and the office, which is made up of two Co-CEOs as experts for the scientific and legal/political aspects and administration who are in charge of the daily business. Since 2013, the operational functions of the latter two are distinguished into 5 different areas (Research, Education, Information, Legislation and Politics), for which they are individually responsible. There is nevertheless a close collaboration and consultation between them and external experts at all times.

FFVFF/AfR has only accepted donations from private parties, never from governmental or industrial sources. The rationale was and is the avoidance of conflicts of interest. The foundation wishes to stay independent in all its funding decisions. In addition, this strategy allows it to present itself to the scientific community and the public as having a sole and exclusive interest in animal welfare and the avoidance of animal experimentation.

1.4.2 Principles of project funding

The rules for funding projects are defined in the “Stiftungsurkunde” as well as the regulation for awarding research grants (Appendix II) and a predefined form for filing applications. Both of the latter can be found at the website of the AfR [3] (English and German). In accordance with the Foundation’s objectives, considered for funding are projects and research, which have the potential to replace or reduce experimental animal numbers.

The most important rule has always been that Animalfree Research will not fund a project involving animal experimentation, regardless of the rationale. Other criteria that are assessed within the application, apart from scientific quality, include e.g. the impact on the 3Rs (animal numbers, animal welfare), the clear intention or motivation to reduce animal numbers and/or suffering, the background of the project leader, and the likelihood of success.

The scientific expert at the office is in charge of reviewing incoming applications and advising project leaders. Receipt of an application is acknowledged by the office within 14 days. The decision for funding is made by the steering committee during the meeting following the receipt of a valid application.

In order to protect donor´s money, precautionary steps are taken in a written contract. A general form is presented in Appendix I. Milestones are defined and the funding money is usually paid only by instalments upon accomplishment of these. In case of continuous and serious breach of contract (e.g. loss of 3R relevant impact), Animalfree Research is entitled to demand the funding sum back (partially or entirely).

The project leader is obliged to report delays or difficulties to the responsible persons at the AfR immediately when they become evident.

Both parties support each other in publishing and implementing the results of the study.

1.4. Terms and definitions

Alternative methods and the 3R concept

In this study, the terms “3Rs”, “3R concept” and “3R principles” are used synonymously, and are understood as approaches that replace, reduce or refine in vivo experiments or improve animal husbandry. It has been attempted to avoid the term “alternative” as far as was possible, since it is misleading: alternative (as in “alternative medicine”) is implicating that it co-exists beside the “old” method as a second option (which is exactly not the point), and may be used or not. This is contrary to the intention of Animal Welfare Ordinance Article 137.2. which prohibits animal use if an alternative method that is suitable is available.

Animalfree Research vs. FFVFF

The foundation was renamed into Animalfree Research in 2007 after decades of being known as FFVFF. In this report, the foundation will uniformly be referred to as FFVFF until 2007, and later as the Animalfree Research (or AfR).

Categorization: academic institutions and industry

In this report, basic (fundamental) science is attributed to the academic institutions and regulatory testing to industry. There are important caveats to this approach. For one, industry not only does regulatory (e.g. safety/toxicity testing) but also basic, academic science. On the other hand, academic institutions do highly standardized laboratory work, using Standard Operating Procedures, and even work according to the principles of Good Laboratory Practice (GLP). Furthermore, the results from basic and especially applied science coming from universities may enter the industrial sector for e.g. validation and commercialization.

The areas are therefore overlapping, and are interacting and supplementing each other. Science can be understood as a process, blurring the distinctions between areas.

The distinction performed here is therefore an oversimplification. Nevertheless, for the sake of clarity and brevity, in this report, basic research is attributed to academic institutions, and regulatory testing to industry.

"3R Research" vs. "non-3R Research"

In this report, there is a differentiation being made between "conventional" or "mainstream" research / science (non-3R Research) and "3R Research". It is nevertheless difficult to address these approaches term-wise.

One has to stress:

That "non-3R Research" is not necessarily animal-based.

That all laboratories in Switzerland using animals nowadays have embraced the 3R concept; in vitro methods are used as pre-screening methods, supplements etc. and wherever possible, fewer animals and less distressful approaches are used.

Quite a few researchers are developing / optimizing / adopting in their laboratory methods that replace / reduce animals, but are not aware that what they are doing is "3R-relevant".

With regards to the term "3R Research", it has been stated that there is no such thing. Obviously, scientists working on a 3R-relevant method do not do so in a special "3R laboratory" in a galaxy far, far away, but certainly use the same equipment and approaches as do all other scientists in the world. The understanding in this report is as follows: research that has the explicit goal to reduce animal use and/or to improve the welfare of laboratory animals, an aim that is equal or even paramount to the scientific goal is referred to as "3R Research"; while project work that has predominantly scientific aims is called "mainstream" or "conventional" science.

"Transgenic" vs. "genetically modified" animals

As it was stated in a Workshop Report from the "Bundesinstitut für Risikobewertung (BfR)" Expert Workshop 2009 in Berlin, there have been inconsistencies in the use of the two above-mentioned terms. The authors state that transgenic animals are only those that carry foreign DNA in their genome, whereas the term "genetically modified" is much broader,

comprising transgenic animals as defined above as well as knock-ins, knock-outs, as well as knock-downs [4]. The Swiss Animal Welfare Ordinance speaks of genetically modified animals ("gentechnisch verändert"), and this term, and the above-mentioned understanding of the term, will be used in this report.

Years

The dates refer to the year of project approval, if not explicitly stated otherwise. A publication dating from 1995 and deriving from a project approved in 1988, for instance, is depicted in graphs under 1988.

Publications

Publications in this report are namely original articles, conference papers, reviews, and PhD Theses. This restriction is due to the fact that publications such as posters and oral presentations proved too intangible (especially after forty years) for a systematic count.

In addition, there is doubt whether in the earlier years of the foundation, they insisted on an acknowledgement of support in a given paper. Since only publications with an explicit written acknowledgement were included, there is the possibility that several of them are missing in action.

The numbers provided in this report are therefore minimum numbers.

1.5 Sources

All information used in this report came from the following sources:

- The project documentation of the AfR / FFVFF
- The AfR website
- Original publications
- Email interviews with team members
- A questionnaire sent out to former project leaders / project members
- Website of the "Federal Veterinary Office FVO"
- PubMed
- Google Scholar
- Scopus
- ALTEX website
- Diverse websites of Animal-Welfare organizations and Federal Offices of Switzerland, Germany, and the United Kingdom

1.6 Limitations

Information on the earliest projects of the FFVFF proved difficult to retrieve, especially due to lack of documentation and/or unavailable or deceased scientists / project leaders. For some projects publications were no longer available as a full online text. The return quote on the

questionnaire, sent out to any available project-partner, was good - but as mentioned before many researchers could not be located. Some of the addressed scientists could no longer recall details of their former projects.

Animal numbers and reduction of severity degree

It proved impossible to determine the number of animals saved or affected by a given project. There are multiple reasons for this, the most important being that:

In a number of cases, the AfR foundation was not the only sponsor, but co-financed a project together with others. It is therefore not possible to determine how instrumental the funding was for the scientific and animal welfare impact of a given project.

Very few methods, when introduced, permit a complete and immediate switch from in vivo to in vitro and provide unambiguous numbers on the reduction of animal use.

Even in these ideal cases, this reduction cannot be extrapolated into an indeterminate future. In the course of the years, in vitro methods (the 3R concept in general) have been widely embraced by the scientific community, and it is impossible to say at what time point the animal experiment would have been replaced or supplemented anyway. The same holds true for other working groups, who adopted the method (if, for that matter, it is at all known who adopted the method and when – and how successfully).

In the case of reduction, where the animal experiment is replaced or supplemented by in vitro methods, quite often more experiments are performed, because the in vitro method is quicker and cheaper. It is therefore not feasible to calculate the reduction of animal numbers according to the experiments performed.

Refinement can affect the experiment itself, in which case a reduction of the severity degree can be assumed. But there are also refinement measures that concern housing and husbandry, aiming for improving the living conditions for the animals. In this latter case, it is not possible to quantify the degree of improvement.

For these and many other reasons, the provision of numbers was considered to be an at best, misleading, but most definitely not scientifically rigorous approach.

2 Projects and their impact

In the following chapters, a multiplicity of backtracked projects, partly or fully funded by the FFVFF or AfR, will be discussed. For each project a brief description, including the aim and the approach of the scientific aspects as well as an objective outcome of the project will be provided. The author tried to follow-up the project up to recent days. If possible, enhancements in methods were tried to be found, in the best case being adapted in an increasing number of laboratories up until today.

2.1 Searching for Replacement of the LD_{50} Test, Gerhard Zbinden (1977-1981)

2.1.1 Project description

As its first project, FFVFF completely funded this literature study, running from 1977 until 1981. The aim of the study was to critically follow up multiple studies using the classical "Lethal Dose 50 (LD_{50})" procedure to recognize the lethal dose of a substance in the field of toxicology. The determination of the LD_{50} was originally introduced as a simple test to obtain general information on the potency or the potential acute hazard of a chemical substance. To find the lethal dose, typically rats or mice (but also rabbits, dogs or monkeys) are divided into 4 to 5 experimental groups of a minimum of 5 (but usually 8-10 or more) animals per dose group. Each group receives a certain dose of the tested compound. The substance is given via oral or dermal application, inhalation or via injection. The different dose levels are selected in such a way that 0% up to 100% of the animals will die. Observation time lasts from 1 to 4 weeks. The particular dose with which 50% of the animals die is then referred to as the LD_{50} dose. This test has been widely used since its development in 1927 and is still being used as a standard test by researchers and also for testing toxicity of some cosmetics and lifestyle products.

Zbinden & Flurys' review study [5] analysed the validity of this in vivo model.

2.1.2 Project analysis

Since the early 1980s, more and more criticisms were made about the LD_{50} test, saying that it was highly unethical to cause a high number of animals to suffer severely due to the high side effects leading to the death of the animals. Zbinden and Flury published their review article in 1981, questioning the validity of the model due to the large number of variable factors. These include: animal species, age of the animal, weight of the animals, sex of the animals, genetic influences, animal health, diet, food deprivation or method of administration. Also, the housing conditions, the temperature or seasonal variations were discussed as factors that could sometimes play a role in the outcome of the experiment.

Zbinden and Flury then concluded that upon their review certain changes should be made in toxicological guidelines by regulatory agencies. These changes included the prohibition of use of large animals like dogs, monkeys and pigs; a reduction of small animals used and a prohibition of conducting the LD_{50} test in new born animals.

Even though the classic in vivo LD_{50} test is still being performed widely in medical research, nowadays it is forbidden to perform this test for the evaluation of cosmetics and other lifestyle products throughout the European Union. Since 2009 it is also forbidden to sell cosmetics which were tested in animal experiments in the EU [6]. In medical research, the number of animals used for LD_{50}-testing is steadily decreasing. Since 2005 three in vitro alternative methods for replacing the in vivo LD_{50} test received approval.

The impact which the Zbinden & Flurys' paper had on further research and the success of critics fighting to reduce the number of animal is hard to reconstruct – which is always the case with any scientific paper.

Scientific impact: according to Google Scholar, the Zbinden & Flurys review article (financed by FFVFF) was cited over 220 times in other scientific papers. A variety of webpages can be

found in which Zbinden is cited or interviewed. For example, the city of Wuerzburg calls him a “worldwide recognized toxicology professor” [7]. This strongly indicates that the review paper was not trivial for further research and might even have had a pioneer and/or exposing function.

Impact on animal welfare: In 1982, Prof. Zbinden gave a television interview in which he criticized the LD_{50} test, both with regards to animal welfare and relevance. This was watched by Hildegard Doerenkamp, a wealthy lady with a big heart who had long been waiting for an opportunity to donate part of her fortune for the welfare of animals. Prof. Zbinden did not see a way to abandon the animal experiment in science altogether, but had subscribed to terminating irrelevant and distressful testing. H. Doerenkamp started to support his work, and the collaboration and discussions between the two subsequently led to the founding of the first Doerenkamp-Zbinden Chair in 1985, and the first University Chair for Alternatives to Animals in Erlangen, Germany. To this day, DZF has founded Chairs for Alternative methods in Erlangen, Utrecht, Konstanz, Geneva, and Tiruchirappalli, amongst many other things.

2.2 The ALTEX Journal

The lack of adequate platforms to publish and distribute alternative methods led, in the year 1984, to the founding of the ALTEX (Alternatives to Animal Experiments) journal, which was entirely a creation of FFVFF. Until 1996 it had invested approx. 1 Million Swiss Francs into its development. It used as an example (again) FRAME and ATLA (see chapter 2.2.1.). The reason why there was a demand for such a journal is one that is still relevant today: the development of new methods designed to replace or reduce live animals is certainly a breakthrough for animal welfare, but not a scientific topic that “classical” journals publish.

ALTEX started out as a twice-a-year periodical (German speaking with an English abstract) and, in 2008, had developed into a full-fledged English-speaking journal, publishing original articles, short communications, reviews, as well as news and comments and meeting reports. The published topics include research on the development and promotion of alternatives to animal experiments and bioethics as well as critical reviews on the relevance of animal experimentation. Today, it is Open Access (“Gold”), appearing four times a year, with an impact factor of 5.824 [8].

In 2006, ALTEX became the Swiss Society ALTEX Edition and is today the official journal of the American Society for Cellular and Computational Toxicology (ASCCT) the Center for Alternatives to Animal Testing (CAAT) in the U.S. and in Europe, the Doerenkamp-Zbinden Chairs, the European consensus platform for alternatives, the European Society for Alternatives to Animal Testing (formerly MEGAT) and the transatlantic think tank for toxicology (t4).

2.2.1 Further support to 3R implementation projects

Next to founding the ALTEX magazine to give the 3R movement a platform to publish animal welfare relevant data, the FFVFF (and later AfR) was multi-present in supporting new ideas. To name the most important ones:

- The FFVFF was significantly involved in the foundation of the **European Research Group for Alternatives to Toxicity Testing (ERGATT)** in 1985.

- Since 1987, FFVFF's annual support of **FRAME (Fund for the Replacement of Animals in Medical Experiments)** helped publish the magazine **"Alternatives to Laboratory Animals" (ATLA)**, the English spin-off of "ALTEX" (see chapter 3.2).
- Also in 1987, FFVFF helped found the "**Stiftung 3R**" in Bern, Switzerland. Similar to FFVFF, the foundation supports research projects inland and abroad (for more information see their homepage: www.forschung3r.ch).
- One year later, in 1988, the members of FFVFF gave the impulse to found the **"Schweizerisches Institut für Alternativen zu Tierexperimenten" (SIAT)**. The idea was to found a Swiss counterpart to the existing European institution "European Centre for the Validation of Alternative Methods" (ECVAM). Due to the lack of financial feasibility, the SIAT had to close again in 1996 but thanks to the Fleitmann-foundation in Luzern, Switzerland, parts of it could be continued in the form of the Biographics Laboratory in Basel, Switzerland (see chapter 3.5.1.).
- Since 1994, the FFVFF regularly helps with financing the **"Mitteleuropäische Gesellschaft für Alternativen zu Tierversuchen" (MEGAT)**, today called **"European Society for Alternatives to Animal Testing" (EUSAAT)** to organize the European Congress on Alternatives to Animal Testing in Linz, Austria. Today this Conference is one of the largest and most important conferences worldwide on alternatives to animal testing.
- Numerous of national and international **workshops** have been organized since the first one in 1984 (with the aim to find valid replacements for the painful "Draize-test", a test performed on rabbits to test the acute toxicity of substances to the eye of the test animal). Until today AfR invites people from animal welfare organisations, scientists and people from the industry to their workshops. This probably has and will help in the search for alternative methods and their implementation in the labs.
- In the year 2001, FFVFF assisted with building up the website **"InterNICHE" (International Network for Humane Education**, www.interniche.org), an open and international network of students, teachers and animal welfare activists, working together to try to reduce the number of animals that are being used in education. Until today, InterNICHE has published an educational video (available in 20 languages), and a book called "From Guinea Pig to Computer Mouse" (Nick Jukes & Mihnea Chiuia, 2nd edition, published by InterNICHE, 2006), describing over 500 advanced products to be used for teaching.

2.3 Model of Epilepsy Using Rat Brain Slices, Helmut Haas (1985-1987)

2.3.1 Project description

In the years 1985-1987 this project, aiming to develop a new in vitro model for epilepsy, was completely financed by the FFVFF. Helmut Haas and his team found that it was possible to use brain slices of rats rather than rats for testing new compounds with possible antiepileptic effects.

Finding a valid in vitro model for epilepsy seems important since existing in vivo models are very stressful and often involve physical interventions. These interventions include (depending on which form of epilepsy is being studied) the administration of substances such as pentylenetetrazol or strychnine, giving electrical impulses into specific brain areas, or surgically implanting recording EEG electrodes to measure seizures. Also, models of specially bred animals, for example Wistar Albino Glaxo/Rijwijk rats (WAG/Rij) or Genetic

Absence Epilepsy Rats of Strasbourg (GAERS), are available as a model for genetic absence epilepsy [9] [10] [11].

One of the most studied, electrically induced in vivo models for epilepsy, and therefore a relevant example of the procedures used on rodents, is seizure-inducing kindling. Via stereotaxic surgery, the mice or rats receive two permanent electrodes, implanted through two holes drilled into the skull and into a certain bilateral brain area called the amygdala (part of the limbic system). After a recovery period, the animals receive repeated electrical stimulation with increasing electrical frequency and amplitude. Kindling refers to a seizure-induced plasticity phenomenon that occurs when repeated electrical stimulation evokes a progressive enhancement of seizure susceptibility. Ultimately, it culminates in emergence of spontaneous seizures and the establishment of a permanent epileptic state [12]. In these animals five different stages of seizure are recognized and potential antiepileptic compounds are administered to determine whether they reduce the severity and/or frequency of seizures.

Since a kindling-conditioned animal can be used for a long period of time (over time, several different compounds can be tested in one animal if a reasonable wash-out period is used) - just like in some other in vivo models of epilepsy – the animals experience distress, pain and discomposure to varying degrees depending on the type of model used. Typically, an experiment contains 6-20 rodents per treatment group, tested in a cross-over design, so that each animal can be used as its own control.

Helmut Haas and his group at the Neurosurgical Teaching Hospital in Zürich, Switzerland, developed a method for which only brain slices of rats are being used, more precisely, slices of the rats' hippocampus. In this method a rat is killed by gassing, the brain removed and the hippocampus cut into several thin slices. By using electrophysiological methods on the slices of hippocampus, several applications of anti-epileptic compounds can be studied, using just one or a few animals for the whole experiment instead of the many more that would be needed for a study in living animals. The use of brain slices from both rats and mice are now quite commonly used in electrophysiological studies of anti-epileptic drugs.

2.3.2 Project analysis

From an animal welfare point of view this ex vivo method is not optimal since animals still have to be sacrificed to isolate their brain tissue. The advantage, compared to the in vivo model is, that one rat's brain can be cut into a multiplicity of slices for testing many compounds or doses. In the best case one rat brain can replace up to 30-40 rats although this may be less if small rat brain regions or mouse brains are being studied because fewer slices can be obtained. The animals do not have the stress and pain of going through surgery and the long procedure of kindling. Next to the benefits for the rodents and the decreased number of animals, the ex vivo model has all the advantages of not being an in vivo model: results are not highly dependent on variance due to age, gender, strain or husbandry conditions.

Today, the hippocampal slice model of epilepsy is well established and widely used in basic and medical research. With 22 citations Haas' publication certainly played its role in this development. After developing and establishing the model, Haas and his group were financed by SET ("Stiftung zur Förderung der Erforschung von Ersatz- und Ergänzungsmethoden zur Einschränkung von Tierversuchen") to train other researchers in

how to use their new technique by offering classes, helping to disseminate awareness and uptake of this method (SET-Project 006, “Lehrlabor zur Vermittlung der Hirnschnitt-Technik”).

2.4 Animal Use in Education

For a study on animal use in German educational institutions, FFVFF provided partial funding to the German “Bundesverband studentischer Arbeitsgruppen gegen Tiermissbrauch im Studium” (SATIS) in 1995. The authors published „Erfassung des Tierverbrauchs und des Einsatzes von Alternativmethoden im Studium an deutschen Hochschulen“ in 2000. The questionnaire-based survey addressed educational institutions with obligatory courses which probably used live animals or organ preparations. 86% of the questionnaires were returned and altogether 262 courses evaluated. In sharp contrast to the Austrian results (please see below) the authors stated in the article’s summary that “it is still hardly possible to graduate in biology, medicine and veterinary medicine without taking part in courses where animals are used”. Of the 60 faculties of biology, 8 did not require obligatory use of animals or offered alternatives, of the 36 medical faculties, 4, and of the 5 veterinary institutions, none. At the same time, according to an earlier survey, 80% of students expressed their wish for animal-free alternatives [13]. Approx. 60’000 animals were found to be killed in the surveyed curricula. Students unwilling to participate were at times denied the certificates of completion. At the same time the authors noted that a large range of alternative methods was already in use and that it seemed to be possible to provide a good education using only those. The article was summarized in the 2000 issue of the book series “Ersatz- und Ergänzungsmethoden zu Tierversuchen” [14].

In 1995 FFVFF provided full funding to another questionnaire-based study by the “Arbeitskreis für die Förderung von tierversuchsfreier Forschung” (AFTF) in Austria which sent questionnaires to altogether 246 institutes located at faculties for human and veterinary medicine as well as the life sciences at Austrian Universities, asking whether they were using animals for educational purposes. The responding rate was 100% (!) and the results somewhat surprising. Only 6 institutions (2.44%) were claiming to use animals for teaching purposes (the majority non-lethal), and 5 of these were offering non-animal alternatives to their students. These findings were in sharp contradiction to those in Germany, where the use live animals were a major part of teaching and training. The study „Tierversuche und tierverbrauchende Methoden bei Pflichtlehrveranstaltungen an österreichischen Universitäten“ was published in the 2000 issue of the book series “Ersatz- und Ergänzungsmethoden zu Tierversuchen” [14].

Ongoing concerns about animal use for educational purposes resulted in the support of two computational programmes which are described below as well as the funding of InterNiche (see 2.2.1) and the Brazilian project (see below).

A recent ranking of university faculties (updated in April 2016) by SATIS conveyed the following information [15]:

	No animal experimen-tation at all+	Animal experimenta-tion can be avoided without disadvantage/ the animal experiment is not distressful	Animal experimenta-tion cannot be avoided/is distressful and/or lethal++	no information available
Biology	6	14	42	4
Human medicine	8	12	15	0
Veterinary medicine*	0	0	5	0

Tab.1: Ranking by SATIS
+ Only alternative approaches or dead animals donated to the university by the owners
++ This includes the use of organs, e.g. from abattoirs, as well as non-vertebrates such as earthworms
*number of courses with obligatory animal use varies between 1 and 6 between universities

2.5 Pharmatutor, Daniel Keller (1986-1997)

2.5.1 Project description

In 1986/87, the years that FFVFF fully supported the development of the software “Pharmatutor”, it was common for students of medicine, pharmacology or pharmacy to use rodents for their education. Testing compounds which change physiological parameters such as blood pressure or pulse helped them understand the responses of an organism.

The idea of the software was to reduce the number of animals (especially mice) used every year for demonstration purposes at universities in Europe.

Pharmatutor is a graphic-based interactive computer programme. It consists of 5 parts, each designed to be a self-contained practical class exercise that can be completed in a relatively short time (20 to 25 mins.):

1. Pharmacokinetic simulations (IV injection, IV infusion, single oral dose, two-compartment model, renal insufficiency);

2. Blood pressure and catecholamines;

3. Blood pressure and acetylcholine;

4. Smooth muscle in an organ bath;

5. Neuro-muscular transmission (including the effects of tubocurarine, suxamethonium and neostigmine in various combinations).

2.5.2 Project analysis

The software Pharmatutor was first presented in 1986 at an international congress for alternative methods in Versailles. According to a statement written by Daniel Keller ("Pharmakologie-Unterricht am Computer") the interest in the project was high and within a short period of time over 20 copies were given to universities for internal distribution. The software was given out as freeware which made it more accessible and contributed to its popularity. The Pharmatutor was published in ALTEX in 1987 and in the ILAR Journal in Volume 38, Number 2 in 1997. An overview published at the 3rd World Congress on Alternatives to Animal Use in Baltimore in 1993 denoted it as the most extensively distributed educational programme worldwide.

In recognition of his pioneering work, Daniel Keller was awarded the animal welfare prize of the French Organization Association de Defense des Animaux de Compagnie.

Today the software Pharmatutor is still available for download [16] but the software has not been extended or revised since 1987. It is therefore out of date, particularly in regards to today's technical possibilities.

It was not possible to find reliable numbers of animals to be sacrificed for educational reasons since the 1980s, and how the amount changed due to the launch of the Pharmatutor during the following years.

In 1995, the same author presented a follow-up, the PharmaSim [17]. PharmaSim (developed over two years at the ETH Zurich and again, free of charge) was a programme capable of simulating pharmacokinetics (drug levels in the plasma) based on compartment models. FFVFF helped the project to take off with financial support in the first year. Both simulators were published on the InterNiche website and were mentioned in a review on worldwide essential software in 2011 [18].

2.6 Virtual Tox Lab, Angelo Vedani (1996 ff.)

2.6.1 Project description

Starting in 1983, Angelo Vedani (today Associate Professor of the Department of Pharmaceutical Sciences at the University of Basel) received regular financial support by FFVFF and AfR to create a tool to virtually predict the toxic potential of chemicals and drugs. In 1990, the FFVFF funding was decisive in the founding of the "Schweizerische Institut für Alternativmethoden" (Swiss Institute for Alternatives to Animal Testing) SIAT, which opened in 1991. The Institute was soon thereafter transformed into the Foundation Biografik Labor 3R. The foundation exists to this day and hosts the "*VirtualToxLab*", which simulates and quantitatively predicts the interaction of a compound with a series of proteins known to trigger adverse effects using automated, flexible docking combined with multidimensional quantitative structure-activity relationships (QSAR). Currently, the *VirtualToxLab* comprises 16 models of proteins known or suspected to trigger adverse effects, including the androgen,

glucocorticoid, liver X, mineralcorticoid, thyroid α and β receptors and several enzymes of the CYP450- family. Most importantly, though, the VirtualToxLab allows rationalization of a prediction at the molecular level by analysing the binding mode of the tested compound towards all 16 target proteins in real-time 3D/4D (Biograf 3R [19]).

Thus, this project aimed to contribute to two aspects of the 3R philosophy: 1.) To reduce the numbers of animals (rodents, but subsequently also dogs and primates) used for toxicological and pharmacological testing to detect side effects of compounds as well as reducing the physical stress and pain by replacing the most severe experiments with this computer based “in silico” model, and 2.) To create a widely used database which would then reduce the number of otherwise double-conducted tests at research laboratories.

2.6.2 Project analysis

FFVFF started supporting the development of this computer-based approach at a time when computer modelling was hardly being worked on by others in the 3R scene. Hence, FFVFF took a brave and forward-looking decision by supporting this new approach – on and off for 24 years (1983-2007).

The development of VirtualToxLab has taken over 15 years, and still continues today since it has to incorporate the new advances in research as they emerge. As well as providing partial financing to this project, FFVFF/AfR also played a major role in finding other bodies to help secure the long-term sponsorship necessary to enable the project to fully develop. Today, parts of the software, the so called “Yeti” and “Quasar”, can be downloaded as freeware and according to Professor Angelo Vedani, they are being downloaded 30-50 times a month.

VirtualToxLab itself can be freely accessed by all non-profit organizations such as hospitals and universities (see www.virtualtoxlab.org) and it is now being used by over 80 institutions worldwide [20]. Since 2008 it can also be acquired by for-profit organizations, and today VirtualToxLab is just cost-effective (e.g. self-financing). Four of the world’s biggest research corporations are using the software today.

Only a very few publications however cite VirtualToxLab. This discrepancy with its commercial success might be due to the fact that the VirtualToxLab software is being used in the very early stage of drug-/compound- discovery and research, which rarely gets published.

Concerning the animal-welfare point of view, the VirtualToxLab probably didn't have a great effect on the number of animals used for toxicological and pharmacological experiments. Since the VirtualToxLab gives out very quick results (compared to its equivalent in vivo studies), scientists use it as an efficient way to screen many more compounds for potential safety liabilities than it would be possible using animals, but in the end the most important compounds are still tested in vivo to confirm the safety prediction.

The most beneficial impact of VirtualToxLab in terms of the 3R objectives has probably been the reduction/elimination of in vivo testing of substances with a strong toxicity potential, and thereby achieving a reduction in animal suffering compared to a situation where VirtualToxLab would not have been available.

2.7 New Fish Cell Line for Ecotoxicological Screening, Karl Fent (1996-1998)

2.7.1 Project description

Karl Fent's project was partly financed by FFVFF for 2 years (1996-1998). Its objective was to establish a permanent cell line which can be used for toxicological screening of wastewater.

Up until today industries use the so-called "Golden orfe test" or "fish test" to assess the toxicity of wastewater which is being discharged into public bodies of water. By law, no toxic water may be introduced into the water so the wastewater is first being bypassed into tanks containing orfes. Survival of 3 fish per tank is monitored for 48h in several different dilutions of the wastewater. The dilution in which no fish perishes is determined and accordingly reprocessed.

Each control sample has to pass through 12 experiments with a minimum of 3 fish and 4 different doses being involved in one single spot check.

Yearly about 40'000 to 50'000 fish were sacrificed for this procedure in Germany alone [21]. In 2011 18'500 orfes were killed in Switzerland [22]. Considering such large numbers of fish being sacrificed for the screening of toxicological wastewater, finding an alternative method seemed very reasonable.

Karl Fent supervised the PhD thesis of Detlev Jung and the master thesis of Daniel Baumgartner, both projects outlining the idea of using the permanent hepatoma-cell-line PLHC-1 (obtained from a Eurasian minnow species) as an alternative to using orfes. They found a correlation between the in vitro toxicity and the acute toxicity of a compound. Additionally, they experimented with the possibility of growing the cell line on a serum free media (see chapter 2.9), thereby helping to reduce the extraction (and use) of foetal bovine serum.

2.7.2 Project analysis

Since the 1st of January 2005, it is forbidden to use fish for eco-toxicological experiments in Germany. In Switzerland there are now several approved alternative methods but fish are still being used. However, since the approval of the alternative methods in 2013, the number of fish has significantly dropped from approximately 18'500 in 2011 to 2'200 in 2013.

Even though not the number one choice of in vitro experiments, with 63 citations on Google Scholar, the paper of Jung, Klaus & Fent [23] probably gave a good impulse to researchers around the world on the importance of finding an alternative to the "fish test". Today we find a greater variety of established in vitro models, such as the "fish egg test" (developed by Roland Nagel at the University of Mainz, Germany), the "daphnia test" or the "algae reproduction test". The often-used permanent cell line RTgill-Wt ("Rainbow trout gill-Waterloo 1") was established by the Swiss research institute eawag (Eidgenössische Anstalt für Wasserversorgung, Abwasserreinigung und Gewässerschutz) – for which Karl Fent was working.

The PLHC-1 cell line can be purchased commercially today and according to one of the providers, ATCC, it can be "used in an in vitro system to screen environmentally relevant stressors such as heavy metals using a combined stress protein and cytotoxicity assay" [24].

2.8 EpiDerm™, Manfred Liebsch (1996-2009)

2.8.1 Project description

Phototoxicity, the negative interaction of a compound with (UV) light, is sometimes observed after applying a substance onto the skin. Hence, all compounds that are applied to the skin (drugs, salves or cosmetic products) need to be tested for this reaction.

The most common way of testing for phototoxicity in the past was the “skin irritation test”, mainly using rabbits for the experiments. 24 hours prior to testing, the animals’ fur is partly shaved, and the test substance is applied to the skin for up to 4 hours. The experimental animals are then individually restrained in a small tube and are irradiated with UV light. The area of concern is then observed for several days or even weeks to see if painful inflammations emerge on the animal's skin. This skin irritation test is initially done on one single animal, but if the animal shows a reaction to the procedure and phototoxicity is suspected, more animals are tested.

This form of testing for skin irritations has been widely used, especially in the cosmetic industry. According to “Ärzte gegen Tierversuche”, more than 5’500 animals had to undergo painful procedures in the EU to test ingredients for cosmetics in 2005. Even though promising new models using alternative in vitro approaches (like the present model) were already available in 2007, about 900 rabbits were nevertheless tested in skin irritation tests in Germany alone (press release concerning Epiderm/-skin, Menschen für Tierrechte – Bundesverband der Tierversuchsgegner e.V., 2009).

Manfred Liebsch and his group received funding from FFVFF / AfR in 1996 and 2009 to support their project to develop a new in vitro test for dermal phototoxicity using a model of reconstituted human epidermis called “EpiDerm”. Other than previous “full-skin” models this approach uses a three-dimensional, differentiated model of the human skin. The skin consists of normal, human-derived epidermal keratinocytes which have been cultured to form a multi-layered, highly differential model of the human epidermis [25]. This tissue is often referred to as “reconstructed human epidermis (RhE)” and its ultrastructure closely parallels human skin. On the basis of this in vitro model of the skin, potential cytotoxicity of a compound due to phototoxicity can be measured without harming any animals. The test material can be applied topically to the EpiDerm skin in different concentrations and irradiated by a sun simulation light (UVA and visible light). Cytotoxicity is determined one day after irradiation in a MTT assay (a colorimetric assay for assessing cell metabolic activity).

2.8.2 Project analysis

Phototoxicity is a serious problem in the process of developing a cosmetic or medical product. All compounds need to be tested to see if they are “photoactive”, meaning they interact with UVA or visible light. Phototoxicity occurs when light is absorbed by the substance, leading to a molecular change causing toxicity and resulting in photodermatitis or other skin irritations. If observed, the development of the new compound must be reassessed and often stopped. Skin irritations appearing on the basis of photoactive compounds are not only harmful and painful for humans but can and did cause pain and suffering in test animals. It does seem reasonable to invest into in vitro approaches, not only to spare the test animals suffering but also to find a model better representative of human skin. Rabbit skin has a

slightly different composition than human skin and not all toxicity findings in rabbits can be extrapolated to human skin.

By using appropriate phototoxic and non-phototoxic test chemicals on the EpiDerm™ system, Liebsch and his group found that the reconstructed human epidermal model can be used in phototoxicity testing in the same way as a full skin model (e.g. in vivo model using rabbits) [26].

Since the RhE is obtained from human skin and then cultivated, this in vitro model for phototoxicity does not only imitate the human skin (like rabbit skin does) but does represent it. Hence, the model is not only suitable for testing compounds used in cosmetic products but also for medical products.

The EU-guideline 2003/15/EG from 2003 specifically integrated new directives regulating the use of in vivo models for cosmetics under the terms of reference 76/768/EWG. It forbids the use of animals for the purpose of developing new cosmetics since the year 2004 and for single components in cosmetics since 2007 (whilst it is still allowed for medical products, though) [27].

This huge success in animal welfare could only be achieved because valid in vitro models had been developed to replace the painful animal experiments.

Again, the exact impact the present project had on this positive development in research is hard to prove. The EpiDerm™ is commercially available to scientists, sold and provided by Mattek Corporation, based in Ashland, Massachusetts, USA. Searching for citations of Liebsch´s original publication, 5 results were found. This is not many, but since the 3-D EpiDerm™ model is EU-accredited since 2007 [28], it is probable that this method is being used all over Europe for the development of cosmetic products. It is also possible/feasible that it is being mainly used in basic research with results in this phase rarely being published.

2.9 Serum-free Cell-Culture Medium, René Fischer (2001-2004)

2.9.1 Project description

In vitro models are becoming more and more popular. Testing compounds on cell cultures saves space, costs and can be more reliable than using entire organisms, which may be subject to restrictions due to age, gender or housing conditions. From an animal welfare point of view, in vitro models are a huge success because animals are spared the stress and pain of an experiment.

To grow cell cultures, the cells need certain growth factors and other nutrients to stimulate their growth, to nourish them and to keep them alive. Typically, foetal bovine serum (FBS) is supplemented to the medium because it contains the dietetics the cells need. Also, for cryoconservation (freezing of cells or tissue by cooling to sub-zero temperatures) FBS is thought to be necessary to keep mammalian cells alive.

The production of FBS is by all means a controversial issue: if pregnant cows are being slaughtered – and it is found that the foetus is older than 3 months – the placenta is opened and a syringe is inserted into the still beating heart of the foetus to extract the blood.

Subsequently, the blood is centrifuged and the plasma is then sold to laboratories all over the world.

In “The Use of Foetal Bovine Serum: Ethical or Scientific Problems?” (2002) [29], the author estimates that about 1 million foetuses are being bled to death every year. This approximation is based on the assumption that 500’000 liter of pure serum are sold every year. Other estimations even talk of up to 2 million foetuses a year [30].

Consequently, even though “in vitro” models are the preferable method to the equivalent “in vivo” model, the production of culture medium still involves the harm of animals.

From an animal welfare point of view, taking blood of a foetal bovine falls under the severity degree of 3, the highest level there is. Thus, for ethical reasons, this procedure should not be performed.

AfR funded this study, investigating the possibility of freezing cells using an artificial medium rather than FBS, in cooperation with the “Ligue Suisse” and the “Zuercher Tierschutz” in the years 2001 to 2004. The objective of the project was to find a replacement for FBS, thus, reducing the sacrifice of foetal bovine as well as refinement (by reducing the pain of the animals).

Investigating an artificial culture medium free of FBS for freezing cell lines would have many benefits for scientists: these media would be more constant in their composition while FBS varies in the number of hormones, enzymes, etc. from batch to batch. This leads to decreased reproducibility of experiments both within a lab and between different labs. Serum generated from FBS can be contaminated with viruses or bacteria. The laborious way in which FBS is produced makes it expensive and prices are strongly dependent on the worldwide demand for cattle. All these challenges could be overcome if an artificial culture medium would be available. This could then be sterile, low in cost and, most importantly, constant in its formulation.

René W. Fischer and his group at the Laboratory of Organic Chemistry, Swiss Federal Institute of Technology in Zurich, Switzerland, tested the adaptation of several commonly used cell lines to serum-and protein-free media. They used the synthetic surfactant “Pluronic F68TM“, known to protect mammalian cells grown in serum-free bioreactors. They found a significant increase in viable cells after thawing the cells [31].

2.9.2 Project analysis

Currently, three main types of culture media can be distinguished: serum-containing medium (with FBS), serum-free medium and serum- and protein-free medium (“free of animal derived components”). Hernandez & Fischer’s research showed the successful cryopreservation of many commonly used cell lines using serum- and protein-free medium.

This can be seen as a great success since FBS does not have to be produced for this method.

According to René W. Fischer, there has been further research to optimize the procedure and the serum-free method is still in use in their lab today. It is now also being used widely among other laboratories who adopted the method and no failed attempts to establish the method in other groups were reported.

But today it is still not possible to use serum-free medium for all cell lines. Talking to a scientist from a large biotechnology company, I have learned that certain cells like neurons and all neuron-associated cells can only be frozen and grown on FBS-containing media.

Despite this, Fischer's research can be seen as a great contribution to the 3R movement and animal-welfare. Even if not all cell lines can be preserved in serum-free media just yet, this new method probably helped to decrease the use and production of FBS and helped to establish a more reproducible way of freezing and growing cells.

2.10 Transgenic Animals and Hypertension, Stingl / Völkel / Lindl (2009)

2.10.1 Project description

In 2009, the three scientists Stingl, Völkel & Lindl published an AfR-supported review paper, investigating the use of genetically modified animals (GMO) in the field of blood pressure research in the last 20 years [48]. They used PubMed to find literature indicating a connection between hypertension and transgenic and knock-out animal models. All English reviews and experimental reports found by certain keywords (like "hypertension transgene animal" or "hypertension knock in/out animal") and published between the year 2000 and June 2007, were examined. In total 115 publications were analysed to determine whether they described a connection between transgenic or knock-out animals and primary hypertension.

2.10.2 Project analysis

Hypertension is a major risk factor for heart disease and stroke. As the first and fourth leading causes of death in the United States, respectively, heart disease and stroke occur in approximately 30% of adults [49]. Numbers in European countries are comparable to the ones in the United States with over 1.9 million deaths in the European Union to be caused by cardiovascular disease [50]. Hence, investigating hypertension and developing hypertension drugs is a worthwhile and profitable field of research.

Transgenic and knock-out mouse models for hypertension have existed since 1990. Until 2001, it was believed that the results from animal experiments could be extrapolated relatively quickly to humans, despite species-specific differences. According to newer publications, though, it is not always clear whether blood pressure changes in GMO are a direct consequence of the genetic manipulation. It rather seems that hypertension does not have a monogenic cause. Furthermore, the technology is best developed in mice, whose physiology of blood pressure is different from that in humans.

The review by Stingl et al. showed that the intention of the GMO approach in hypertension did not yet provide any indications for possible applications of the results nor has it provided any basis for human diagnostic or therapeutic application. None of the publications that they examined contained indications of direct application of results gained by using GMO, whether in humans or animals. Considering that this summarizes the results of 20 years of research, this conclusion is rather surprising and quite a knockdown for the research using GMO in the field of hypertension.

Searching Google Scholar for the publication showed only 2 quotes. Therefore, the results of the review did not get the attention it might have deserved, revealing quite some deficiencies in this field of research.

More recently, critical reviews on other diseases revealed that this view is not an isolated finding. Indeed, it seems that the use of animal models for complex human diseases appears to be increasingly questioned. Critical reviews on translational issues with regards to GMOs have appeared recently on Diabetes Type II [51] and on Alzheimer´s Disease [52]. It may be the case that the review of Stingl and Voelkel came too early to be considered by the scientific community.

2.11 Teaching Material for Brazil, Thales de A. Tréz (2008)

2.11.1 Project description

For many years, the organisation “InterNICHE” (also see 2.2.1) has tirelessly worked on its goal to replace animal experimentation in education. Supported by Animalfree Research and others, representatives from InterNICHE are travelling around the world in order to show universities and other educational institutions alternatives to animal experiments and to help implement these methods. Specifically, in eastern European and Latin-American countries animal use for the education of high school students, as well as biologists or veterinarians remains a common approach. This is an unsatisfying situation considering that in this particular area, a myriad of alternative methods is available. The book previously published by InterNICHE «From Guinea Pig to Computer Mouse» [53] is illustrating the entire spectrum of available animal-free methods.

By supporting a new book project of InterNICHE Brasil, the AfR foundation aimed to counteract the use of animals at Brazilian high schools and universities. The book [54] intends to find the underlying cause of the set of problems created by the use of live animals in education, viewing it from different professional perspectives. Its goal is to provide backup and support the tour through Latin America for the distribution of alternative methods.

2.11.2 Project analysis

One important goal was already achieved in Brasil: on the level of junior high schools, animal experiments are now prohibited. But another significant target remains to be met: the ban of animal experiments in higher education. AfR has aimed to achieve and accelerate this with the support of this project.

2.12 Nanotechnology, Ursula Sauer (2008-2011)

2.12.1 Project description

In this AfR - supported project of Ursula G. Sauer, carrying the title “Animal and Non-Animal Experiments in Nanotechnology – the Results of a Critical Literature Survey” [32], a literature study was conducted. There was a total of 164 articles retrieved, all examining in vivo nanotechnological research and covering a) the health care area (e.g. study target drugs, vaccines or gene delivery), b) imaging technologies, c) the toxicity of nanomaterials, d) tissue engineering for regenerative treatment and e) magnetic tumour thermotherapy.

Nanotechnology is science, engineering, and technology conducted at the nanoscale, which is about 1 to 100 nanometers [33]. Due to their small size, nanoscale particles exhibit novel physical characteristics compared to the same bulk chemical without nanoscale features. Nanomedicine is simply the application of nanotechnologies in a healthcare setting and the majority of benefits that have already been seen involve the use of nanoparticles to improve the behaviour of drug substances [34]. Nanomedicine is also a strategic issue for the competitive position of the healthcare industry in Europe. During the first four Calls of EU Framework Programme 7 in the years 2007-2010, the NMP Programme invested about 265 Million Euro in nanomedicine related research projects [35].

As in all fields of medical research, new methods, compounds and substances are widely tested in animal models first. Many of the reviewed experiments were classified as moderately and even severely distressful to the animals. Therefore, the present study tried to answer the question whether such animal experiments are truly the only means to answer the scientific questions addressed in nanotechnology or if there are sufficient in vitro alternatives.

2.12.2 Project analysis

With a large proportion of the articles addressing malignant cancer, tissue engineering for regenerative treatments and the toxicity of nanomaterials, the scientific topics are of high scientific importance.

The results of the literature survey showed that about one fourth of the biomedical research published in scientific journals had been performed *in vivo,* the rest in *in vitro* experiments. The author raises awareness to a probably much higher amount of animal experiments being performed, but not being published (toxicology studies, follow-up *in vivo* studies after finishing the *in vitro* studies, etc). For her there is already sufficient ground for serious concern from the point of view of animal welfare, not only because of the amount of animal experiments being performed but also because of the severity of the experiments. Since the nanotechnology research is a young field of science, Sauer stresses that it was time to design new research strategies that move away from animal experimentation altogether and to base scientific progress on non-animal testing strategies instead.

Having 13 quotes on Google Scholar, Sauer's review seemed to have attracted some attention.

2.13 ALICE-CLOUD, Otmar Schmid/Anke-Gabriele Lenz (2009)

2.13.1 Project description

To detect the effect of short or long-term inhalation of toxins on the organism, primarily rodents are being exposed to polluted air. To observe the toxicity of a substance, healthy animals are placed into an inhalation chamber and exposed to the possible toxin for a defined period of time and then observed for abnormalities. Inhalation experiments are also performed in medical research, where possible cures are tested on artificially diseased animals. To this aim, toxins may be injected into the lung (such as LPS or silica to simulate inflammatory lung diseases), or lung parts damaged either mechanically or by radiation, and

the animals are then exposed to aerosol substances using an inhalation chamber. Afterwards the animals are observed and/or dissected.

Inhalation experiments are very stressful, harmful and painful to the rodents, and, as a screening method for human drugs, not totally suitable due to the physiological lung differences between rodents and humans. Not everything that harms a rat's lung will also be harmful to a human lung and vice versa. Finding an alternative method closer to the human physiology is therefore desirable.

For this reason, AfR contributed to funding the development of the "Air-Liquid Interface Cell Exposure System", in short ALICE-CLOUD, in 2009. This in vitro test uses human lung epithelial cells to test aerosol toxins or compounds [36]. The aim of the project was to find an easy-to-use, widely applicable method which will reduce the use of animals for the respiratory field of research (including basic research, research & development and diagnostics).

2.13.2 Project analysis

In 2005, 87'547 animals, mainly rats and mice, were involved in tests in the respiratory field in the UK alone! A standard toxicology test uses about 90 animals per poisonous substance whilst experiments with mechanical injuries of the lung use 26 rodents per assay. To assess the benefit of a compound on inflammatory reactions after LPS or silica injections, about 210 rats or mice are used per experiment [37]. The ALICE-CLOUD and, respectively, its follow-up model "ALI" could be an effective replacement for all of these time-consuming experiments. Since the commercial launching of ALICE-CLOUD/ALI in 2013 by the company VITROCELL®, a double-digit number of systems have been put into operation. The system can also be purchased in different variations. "The annual statistics of scientific procedures on animals" in Great Britain states that the number of animals (mainly rats and mice) decreased by more than half from 2005 (87'547) to 2014 (41'293) but also within only one year from 2013 (87'651) to 2014. The number of sold ALICE-CLOUD systems so far is probably not a sufficient explanation for this massive decrease of animal experiments within the research field of respiratory diseases. Cited 72 times in publications found in Google Scholar, it is apparent that Schmid's research did indeed have an impact on further research, and that a rethinking is taking place, putting alternative models much more into focus. If numbers of animals keep decreasing to this extent, this will be a huge success for the 3R movement.

According to Tobias Krebs, managing partner of VITROCELL®, the FDA shows interest in the method, which would immensely help to popularize the system.

2.14 New Method for Screening Tetanusvaccine-Toxicity, Karin Weisser / Beate Krämer (2011-2012)

2.14.1 Project description

Especially during the 19th century, tetanus was a widely spread infection, resulting in severe muscle spasms and death in humans. The infection is caused by the bacterium *Clostridium tetani* which usually penetrates into the organism through open wounds. The bacteria then

produce a toxin which interferes with muscle contractions. Today, there is a vaccine to protect humans and animals from this infection. The toxin is manufactured, chemically inactivated and used for immunization. By European law, each batch of toxin produced must be tested on animals to confirm the safety of the product. Mainly guinea pigs and rabbits are used for this toxicological testing. Each batch to be used as a vaccine for humans must be tested on a minimum of 15 animals, batches purposed as animal-vaccine, on a minimum of 10 animals [41].

In Germany alone about 2'000 animals are used for these purposes every year. The severity of the procedure varies from low to high, depending on the presence of residual toxicity in the test sample.

In the years 2011 and 2012, AfR partly financed the optimization of an *ex ante* developed test which was believed to be able to discriminate between toxic and nontoxic batches of tetanus toxin and results from this work were published by Karin Weisser and Beate Kraemer in "Binding and cleavage (BINACLE) assay for the functional in vitro detection of tetanus toxin [42].

2.14.2 Project analysis

In 2010, before being supported by AfR, developments in this project already received some prestigious prizes in Germany, such as the "Tierschutz Forschungspreis" of the Federal Republic of Germany and the "Tierschutzforschungspreis" of the Federal Paul Ehrlich Institute in Langen, Germany.

Looking at the numbers of guinea pigs and rabbits that suffer in safety testing every year, it seems reasonable to look and to invest into new protocols to find alternatives to the existing procedures. The aim of the new method would be to replace the existing animal experiment, used for quality control, with a method which does not involve any animals at all.

At the time of writing this report the project has not commercially developed, according to Weisser & Kraemer, but they are running an international collaborative study together with the European Directorate for the Quality of Medicines and Healthcare (EDQM). Twenty other international labs are currently testing the method and results are expected by the beginning of the year 2016. So far, the findings look promising and further actions for implementing the new method are being planned.

The test has been successfully adapted to botulinum toxin, and was successful in testing three manufacturers´ products. It has therefore high potential to replace the LD_{50} acute mouse toxicity test (published at the Linz Congress 2016).

2.15 New Method of Assessing Pain in Rabbits, Matthew C. Leach (2011)

2.15.1 Project description

Behaviour observations of animals post-procedure are a well-established method for pain assessment in laboratory animals. Pain can occur after surgery or other invasive procedures were performed, or as a side-effect of treatment with compounds. By observing the animals over a period of time, it is thought that experienced and well-trained ethologists are able to

recognize signs of pain in animals such as mice, rats or rabbits. Animals express pain in different physical forms, including decreased activity, abnormal postures (hunched back, writhing, rigidity), poor grooming, weight loss, increased respiratory rate, physical response to touch, teeth grinding, diarrhoea, tremors and more [43]. However, there is little objective evidence to support the commonly used methods of assessing pain. Prior to this study it had never been assessed where and how observers should focus their attention when recording animal behaviour, even though a successful assessment depends on the observer knowing which behaviours to record and where to correctly observe it. With 13'000 laboratory rabbits used in the UK in 2006, of which a large proportion underwent at least one potentially painful procedure during their lifetime (including neutering, orthopaedic and soft-tissue surgery, safety testing and irritancy studies), having a valid method for pain assessment would be of high value for animal welfare [44].

The aim of this study, completely financed by AfR in 2010-2011, was to identify how observers focus on rabbits when trying to assess their post-procedural pain using behavioural-based indices, and whether this influences their effectiveness. A secondary aim was to identify how the experience/background of the observer influences their observation patterns and effectiveness of pain assessment. Since thousands of rabbits are involved in painful animal experiments worldwide every year, it is important to reliably know the signs of pain in rabbits and where they occur in order for the experimenter to draw conclusions and take correct actions.

The present study analysed historical video recordings of white rabbits (*Oryctolagus cuniculi*), which were considered to be experiencing varying degrees of postoperative pain, to assess the pattern of behaviours and the ability to assess pain exhibited by experienced and inexperienced human participants. Eye tracking equipment was used to identify how quickly, how frequently and for how long different areas of the rabbit's body were observed [45].

2.15.2 Project analysis

By analysing eye-movement and pain scoring in 151 experienced and inexperienced participants, Leach and his group concluded that observers focused on the face of the rabbits when assessing pain by behavioural conspicuousness. Experience had no impact on the effectiveness of scoring pain. Focusing on the face, though, is unlikely to be effective when using behavioural indicators of pain since they involve other body areas. Validated behavioural-based pain assessment in rabbits have demonstrated that the behaviours and postures considered indicative of pain are predominantly specific to the type and location of the potentially painful procedure.

As a direct impact of this study, observers can now be trained on localizing pain in rabbits more precisely, not only concentrating on facial expression in rabbits (and possibly also other laboratory animals), and thereby improve efficiency of observation and reduce severity in animals. Thus, this study aimed on refinement of experiments using rabbits.

According to Leach the method has been adapted by other working groups and an estimated >300 people were trained according to the findings of this publication. The method is currently being further optimized with publications currently being drafted.

The 21 citations on the AfR-supported paper at Google Scholar and 5,530 views and 41 saves on PLos ONE underline the success of this project.

2.16 Intracellular trafficking of nanoparticles, Barbara Rothen-Rutishauser (2009-2010)

2.16.1 Project description

By using a 3D-cell culture model of the human alveolar epithelial tissue barrier in combination with the newly developed Air-liquid interface cell exposure (ALICE) system (i.e. precursor version of the now commercialised CLOUD system - see above, Project 2.11.), a system to assess the hazard of inhaled aerosols could be applied mimicking the situation in the human lung. In this particular project, the effect as well as the interaction of different gold nanoparticles with human lung cells was evaluated systematically using a stereological method that was, at the time, unique. For example, the influence of the surface characteristics of the nanoparticles on the subsequent uptake into the cell and intracellular fate, i.e. trafficking into various cell organelles, was evaluated.

AfR contributed 11´000 Swiss Francs for equipment and travel costs for the collaboration with the Helmholtz Center in Munich to optimize the system to study the effects of nanoparticles aerosolized on lung cell cultures, thus supporting the aim to reduce and replacing animal experimentation in the field of inhalation toxicology, and at the same time expanding the uses of ALICE. This work was an essential part of the PhD Thesis by Dora Christina Brandenberger and also highly relevant for the academic success of Prof. B. Rothen-Rutishauser which was at this time a group leader.

2.16.2 Project analysis

To this day, the ALICE as well as the commercially available system, i.e the CLOUD, are in use for various national and international projects in the working group of Prof. Rothen-Rutishauser, now located at at the Adolphe Merkle Institute, University of Fribourg, , Switzerland.

2.17 Artificial Gastrointestinal Mucosa, Sara Lindén (2010-2013)

2.17.1 Project description

Gastrointestinal (GI) diseases are becoming more and more common due to a change in lifestyle. Eating habits have changed towards an unhealthy diet with fast food and unbalanced calorie intake. Other factors like stress or smoking may also support the development of (chronic) inflammatory bowel diseases such as Crohn's disease. Studies have shown that the number of children in the Northern and Western European countries suffering from Crohn's disease tripled since 1996. An estimated 2,5 to 3 million Europeans are afflicted with a chronic inflammatory bowel disease (IBD) [38]. As a consequence, a substantial basic research effort and search for new medicine is underway in academia and industry.

Numerous in vivo studies were and are being performed to understand the mechanism leading to inflammation in the gastrointestinal-system and to test compounds that may provide more effective treatments. Many in vivo models of Crohn's disease include a (long-term) exposure to either bacteria (like *Escherichia coli* or *Citrobacter rodentium*) or parts of their membranes (LPS) to induce inflammation which can then be used as a test system to

identify compounds that are able to ameliorate the pathology. For the animals, mainly mice (but also rats, rabbits, sheep, fish or birds), this can be a very stressful and painful procedure since the inflammation causes cramps and diarrhoea. After a specified time (hours to several weeks), the test animals are sacrificed and the intestines dissected and analysed.

The animal experiments are suboptimal for ethical reasons and also because most pathogens cause a different pathology in animals than in humans. Because human pathogens commonly have adhesins for human carbohydrate structures, it is important to select appropriate models for individual pathogens. For example, the effects of *Helicobacter pylori* (*H. pylori*) infection on the mouse are mild, and gastric cancer is not induced even after long-term exposure without other stimuli or genetic defects, although the mouse may develop chronic atrophic gastritis. Similarly, *H. pylori* can colonize the guinea pig and the Mongolian gerbil and cause a severe inflammatory response but does not induce cancer in the absence of exogenous chemical carcinogens. These small animal models are therefore useful to study some aspects of *H. pylori* infection and have the advantage of being relatively cheap. In contrast, rhesus monkeys naturally have persistent *H. pylori* infection leading to loss of mucus, gastritis, gastric ulcers and even cancer. In addition, the anatomy and physiology of the GI tract of the rhesus monkey, as well as the expression of mucins and mucin glycosylation, are very similar to that in human. However, this model is expensive, the monkeys can have pre-existing natural infection, and primate research involves a higher level of ethical considerations. [39]

Sara Lindén and her group received financial support from the Animalfree Research foundation from 2010 to 2013 for development of artificial gastrointestinal mucosal surfaces. The idea was to improve the in vitro cell culture so it resembles the human gastrointestinal mucosa. Specifically, they investigated mucin expression on the apical surface and, on that basis, aimed to study the host-pathogen interactions at the mucosal interface. Succeeding in this aim could result in a high amount of refining and reducing of animals being tested in this field and might even lead to some replacement because the model could be used in many types of research involving the gastrointestinal tract.

2.17.2 Project analysis

Although gastrointestinal human cell lines which produce mucins or polarize already exist, there was a lack of models which reproducibly create the combination of polarized epithelial cell layers, functional tight junctions and a thick adherent mucus layer. This made the development of a more advanced model necessary. Lindén et al. developed a method using standard laboratory equipment that can be used to alter the differentiation state and morphological organization of several cell lines so that it fulfils the above-mentioned requirements. They tested a range of 14 human cell lines and developed an in vitro model of mucosal surfaces suitable for studies of host-pathogen interactions [40].

According to Sara Lindén, the AfR support came at a critical time point of her research and thanks to the endorsement the researchers were able to purchase a cell culture incubator. This device was and is essential for all the in vitro work and the development of a better in vitro model. The in vitro model works so well that Lindén and her group have not used any animals for their research in the last 2 years. They also helped 6 groups set up the model and several other groups are using it (even though there seem to be no publications describing the protocols to date). The cell line cannot be commercially purchased but Lindén

will send the cell line to any researcher that asks for it at no cost. However, some difficulties in reproducibility have been reported by at least one other group.

Still, Lindén's new approach of using human cell lines and developing a method to have a suitable model for mucosal research seems to go in the right direction and other scientists are showing interest in the model. Google Scholar provides 13 citations for the AfR supported publication (with follow-up publications being shortly before publishing) and PLOS ONE additionally shows 10,047 views and 60 saves. This indicates a high amount of interest and attention of other scientists towards the research of Lindén.

Looking at the statistics for animal use in Great Britain over the last years, numbers seem to have promisingly decreased in the field of alimentary/gastrointestinal system. In 2011 a total of 64,560 animals, of which the majority of 48,817 were mice, were used in this field of research. In 2014 the numbers declined to 23,655 (with 16,639 of it being mice). Unfortunately, similarly precise numbers, classified into the field of studies, could not be found for Switzerland or Germany. If the massive reduction of in vivo use in the UK is the result of an increase in in vitro studies or if there was a strong decrease in research done in the area of gastrointestinal diseases could not be ascertained with absolute certainty. However, having valid, easy to use and cheaper alternatives to animal models most probably has an impact on the number of animals used in research.

2.18 New Model of Epilepsy Using Human Brain Slices, Mark Cunningham (2013-2014)

2.18.1 Project description

In 2012-2013 Animalfree Research once more (partially) supported an investigation in the field of epilepsy (see 3.2. Model of Epilepsy using Rat Brain Slices). The validation of an in vitro model using brain slices of rats in the 1980s and 1990s gave a useful alternative to animal models of chronic epilepsy, which were and still are highly stressful for the animals. They involve the repeated application of toxins, invasive surgical methods, and/or use of genetically modified animals (see 3.2), leading to repeated (chronic) episodes of seizures. However, using rat brain slices still meant harming of animals, e.g. sacrificing rats to collect the brain material, even if fewer animals would be needed and substantially less stress would be involved.

For anti-epileptic drug (AEDs) discovery, which necessitates screening of large numbers of compounds, animal models should ideally be easy to perform, be time- and cost-efficient, and predictive of clinical activity. This explains that two simple seizure models in mice and rats, the MES (maximal electroshock induced test) and s.c. pentylenetetrazole (PTZ) tests, which have been developed over 60 years ago, are still the most widely used animal seizure models employed in the search for new AEDs. In the MES test, tonic–clonic seizures are induced by transcorneal or, less often, transauricular application of a short (0.2 s) supra-threshold electrical stimulus in normal mice (50 mA) or rats (150 mA). The endpoint in this test is recording of tonic hindlimb extension, and the test is thought to be a predictive model for generalized tonic–clonic seizures. In the s.c. PTZ (or metrazol) seizure test, the convulsive dose of PTZ inducing a clonic seizure of duration of at least 5 seconds in 97% of the animals is subcutaneously injected and animals are observed for a post-injection period of usually 30 min for the occurrence of such a ‘‘threshold’’ seizure. The test is thought to be

predictive of anticonvulsant drug activity against nonconvulsive (absence or myoclonic) seizures. However, various AEDs that protect against nonconvulsive seizures in epilepsy patients did not significantly affect seizures in the PTZ test [46].

The project of Mark Cunningham of the University of Newcastle had the goal to refine the existing human epileptic in vitro model so it fully captured the profile of electrographic events of the epileptic human electroencephalography (EEG), using electrophysiological, neuroanatomical and in silico computational techniques. About 30% of patients suffering from temporal lobe epilepsy have seizures that are resistant to drug therapy, and many of these are offered surgery to remove the part of the brain where the seizures initiate. Human brain tissue from such surgeries was collected and used for this project. The ability to conduct detailed scientific studies on human brain tissues from patients actually suffering from epilepsy allows a unique insight into the disease.

A positive study outcome will have a promising effect on future research because today.s widely used in vivo models (mainly using rats and mice) have important limitations: a) none of them model idiopathic epilepsy; b) the electrophysiological behaviour is epileptiform and does not capture patterns observed in humans (e.g. face validity of the animal models not optimal), and c) none of the animal models adequately capture drug resistant epilepsy. Using human tissue is probably critical for successful research since the mechanisms underlying epilepsy may be fundamentally different in the human brain as compared with animal models.

Hence, finding a model closer to the human disease situation could have a high effect on replacement, reduction and refinement of in vivo models, which are still being used in basic and medical research. According to Cunningham, his lab annually used about 400 animals (Wistar rats and C57BL/6J mice) for returned procedures of which about 50-100 were used for studies regarding cortical epilepsy. 100% of these animals could become redundant and be completely replaced by 20-30 sets of human cortical slices. Teaching the new method to other groups working on epilepsy could lead to a reduction of animals being used. Furthermore, in a cooperation with the US of A, the researchers aimed to strengthen existing cortical computational models and through this refinement of in silico methods eventually replace in vivo and in vitro models in the future.

2.18.2 Project analysis

Epilepsy is a serious and common chronic neurologic disorder characterized by recurrent seizures, which are caused by abnormal synchronized neuronal discharges. As many as 6 million people in Europe currently have active epilepsy. This has major implications not only for health, but also for independent living, education and employment, mobility, and personal relationships [47].

In 2011 a total of 420’127 animals were used for research on the nervous body system in the UK alone. The exact number of animals used specifically for research on the field of epilepsy could not be found.

At the time of writing, a scientific review paper has been submitted to ALTEX: Limits to the Use of Animal Epilepsy Research Models: Can Epileptic Human Tissue Provide Translational Benefit?

2.19 An animalfree mycetoma grain model to study the therapeutic efficacy of various antifungal agents against the clinical entity of this infection. Wendy van de Sande (2014-2015).*

2.19.1 Project description

Mycetoma is a mutilating, granulomatous, progressive disease endemic in the (sub-) tropical regions. Like many others, it is a neglected tropical (orphan) disease, with the fungus *Madurella mycetomatis* the most commonly encountered pathogen. A feature which all causative agents have in common is that they organize themselves in granules called grains, which can only be formed inside the body – in research, this means in live experimental animals (mice, guinea pigs, monkeys). The severity degree and the mortality are high. An alternative mode was generated using the larvae of the wax moth, which can be used to study the pathology of Mycetoma as well as to test novel diagnostic assays and therapeutic strategies, reducing the number of vertebrate animals needed.

2.19.2 Project analysis

In this study it was determined if the larvae of the greater wax moth *Galleria mellonella* could be used to induce grain formation when infected with *M. mycetomatis*.. At all inocula tested, grains resembling those formed in human and other mammalian hosts were formed within 4 hours after infection.
By developing a model in which grains can be formed outside the mammalian body, a screening tool was generated, with only the most promising therapeutic agents systematically evaluated in animal models. The feasibility of this model system for mycetoma had already been demonstrated in a pilot experiment in which indeed fungal grains were obtained. The model was further refined including other mycetoma species to test novel antifungal strategies.

By generating this model system, the number of animals needed to evaluate a therapeutic response could be reduced considerably. At the time of writing (June 2018) the model was still being used. The group performed antifungal pharmakinetiks/pharmacodynamics in it and demonstrated that the targets of the model are similar to those in humans. A comparison of therapeutic outcomes (efficacy) in the model was compared to historic outcomes achieved in the past in mice, showing satisfactory similarities. The model was also used in a large drug discovery programme (mycetOS, https://www.dndi.org/diseases-projects/open-innovation/mycetos/) to identify the most potent hits. Additionally, it was successfully transferred to other working groups.

* this project caused the board of Animalfree Research to re-evaluate part of the funding strategy. The foundation no longer supports projects that use any animal species for research purposes.

2.20 Supported Academic Studies

The FFVFF and later AfR supported a variety of graduate theses over the years of its existence. Assistance was given by partially financing the scientific work but also by offering expertise and knowledge. The scientific impact of academic studies is difficult to assess

since a thesis very often is only a small piece of work in a flowing process of many researchers. Therefore, some of the work will just be described without any further analysis:

- Hildegard Kohlauf Albertin:“Die Interaktion des Neurophysenhormons Oxytocin mit myometrialen Zellen des Schafs” ETH Zürich 1988 [56]. This work assessed the use of uterus tissue of female sheep, collected from slaughterhouse waste, for testing hormone supplements. Commonly, these compounds are tested on rat tissue and typically the rats are specifically sacrificed to get the tissue.
  In this thesis, Kohlauf Albertin has developed a system which can be used to test the mechanism of action of oxytocin. This new method, using sheep tissue, could be used as an initial screening test for uterotonic substances.

- Kristina Peters: “Experimentelle Untersuchungen zur nichtinvasiven Gewebeablation durch hochenergetischen fokussierten Ultraschall (HIFU)” [54]. This was a PhD thesis for obtaining the degree of doctor in the field of animal medicine at the faculty for veterinary medicine, Ludwig-Maximilians-University in Munich (2007). The thesis of Kristina Peters aimed to use the isolated perfused porcine kidney model to elicit answers to fundamental questions about high-intensity focused ultrasound (HIFU). In oncology, the use of HIFU was becoming increasingly appreciated as a completely non-invasive therapy as a valid alternative to conventional surgery.
  Peters’ findings show that using HIFU in kidney tissue can induce precisely circumscribed homogeneous lesions with irreversible cell devitalisation. It proved possible to control lesion size and also ablate clinically relevant tissue volumes.

- Dora Christina Brandenberger. Interaction of Engineered Nanoparticles with the Respiratory Epithelium in Vitro: Cellular Uptake and Effects. Bern, Switzerland (2010).
- “Organotypic *in vitro* skin models of human cutaneous squamous cell carcinoma” [55]. Dissertation of Suzan Commandeur, Department of Dermatology of the Leiden University Medical Centre, Netherlands (2013). The aim of this research was to develop a representative *in vitro* model of human squamous cell carcinoma (SCC) for screening potential therapeutic compounds, without the unnecessary use of animals. SCC comprises about 15% of all skin cancer diagnoses.
  The author generated several three-dimensional *in vitro* SCC models in which the malignant epidermal cancer cells were either represented by intact primary human cutaneous or by established, spontaneously immortalized human cutaneous SCC cell lines. The dermal microenvironment in the model was seeded with either primary normal human dermal fibroblasts or primary fibroblasts associated with SCCs. In order to test the applicability of this new *in vitro* SCC model as a drug screening tool, it was validated with active compounds, with promising results, indicating that this skin cancer model adds to the spectrum of available *in vitro* models for therapeutic screening.

- Ellen van den Bogaard. From skin development to disease pathogenesis and therapeutics. The power of 3D skin models. Nijmegen (Netherlands) 2014. The goal of the thesis was to develop and apply 3D skin models for epidermal development, wound healing, skin inflammation and therapeutic intervention.The models allowed to reveal the molecular mechanism of coal tar therapy for atopic dermatitis.

Ongoing projects (2016)

2.21 3R-update: A Novel Online Seminar for Literature Search and Publication, Sylvie Vullioud (2014-)

Information literacy is the ability to know when there is a need for information, and to be able to identify, locate, evaluate, and effectively use that information for the issue or problem at hand. Fundamental 3R information is hard to find, because of large dissemination and accessibility difficulty. Therefore, special attention should be given to develop specifically 3R information literacy knowledge to biomedical fundamental researchers.

The aims of the Sylvie Vullioud's project were to train biomedical fundamental researchers located in Switzerland in 3R information literacy, to promote better applications of 3Rs, to improve biomedical reproducibility of results, and to contribute to better biomedical validity in general. The project uses online courses and workshops in already existing mandatory 3R courses organized by LTK (Labortierkunde, Zürich University) accredited by the European FELASA organization (Federation for Laboratory Animal Science Association).

The project 3r-update (www.3rupdate.ch) had in 2016 become a mandatory part of the course for students and researchers involved in animal experiments and provides a comprehensive background on data banks, search strategy, documentation of search, reporting to approving authorities, reproducibility, or ARRIVE Guidelines. So far, over 200 study directors, doctoral students and researchers have participated in the project, and it can thus be considered a success. The report of the project is available at the Zenodo research data repository (https://zenodo.org/record/53166#.V9_u8liLRaQ).

2.22 Development of a Kit for Testing Bone Replacement Materials, Daniel Seitz (2012-)

The goal of this project was to further develop and standardize in vitro characterization methods to a robust test system that can be used as a replacement for animal experiments in the development of bone replacement materials in the research phase. The approach is based on a comparative study of in-house reference materials (calcium phosphate ceramics) and commercial, medically established bone replacement materials whose clinical efficacy has been demonstrated by studies and experience.

The first step was the standardization of cellular tests for mineralization and osteoclast activity. In the next step, analytical methods were applied to co-cultures in which the cell types interact in a simulated bone system.

According to the research leader, the project has been delayed considerably due to the extensive changes at the institute, and the original timetable could not be met. Nevertheless, the examination system and the kit in its present state was presented at the Medica exhibition on 12. - 15. November 2014 at the joint booth of Bayern Innovativ in the context of the presentation of the University of Bayreuth.

3 Publications

All publications were examined whether they bore an acknowledgement for the Foundation. Only those were included into the number of publications which exist due to the support of the AfR foundation. Nevertheless, there are limitations to this assessment since it is possible that in the early times of the Foundation acknowledgements were not included. Furthermore, it might have been difficult to publish results in a peer-reviewed journal. In the beginning of the Foundation, there were simply only very few journals available that would publish articles on 3R-relevant methods. As a consequence, it is therefore possible that the number of publications is underestimated.

3.1 Total number of Publications and Citations (source: google scholar, accessed 14th of February 2018)

Publication	Number of citations
Zbinden G., Flury-Roversi M. (1981): Significance of LD50-Test for the Toxicological Evaluation of Chemical Substances. Archives of Toxicology 47: 77-99.	**291**
Vedani A., Dobler M., Smiesko M. (2012): VirtualToxLab - A platform for estimating the toxic potential of drugs, chemicals and natural products. Toxicology and Applied Pharmacology 261: 142-153.	**54**
Vedani et al. (2009): VirtualToxLab (TM) - In Silico Prediction of the Toxic (endocrine-disrupting) Potential of Drugs, Chemicals and Natural Products. Two Years and 2,000 Compounds of Experience: A Progress Report ALTEX 26: 167-176.	**35**
Vedani et al. (2008): VirtualToxLab - in silico prediction of the endocrine-disrupting potential of drugs and chemicals. Chimia 62: 322-328.	**14**
Vedani et al. (2007): VirtualToxLab - in silico prediction of the toxic potential of drugs and environmental chemicals: Evaluation status and Internet access protocol. ALTEX 24: 153-161.	**20**
Keller D. (1995): Pharmacokinetic Simulations for Teaching. ALTEX 12: 152-155	**1**
SATIS (2000): Erfassung des Tierverbrauchs und des Einsatzes von Alternativmethoden im Studium an deutschen Hochschulen	**5**
AFTF (2000): Tierversuche und tierverbrauchende Methoden bei Pflichtlehrveranstaltungen an österreichischen Universitäten	**2**
Jung D., Klaus T., Fent K. (2001): Cytochrome P450 Introduction by Nitrated Polycyclic Aromatic Hydrocarbons, Azaarenes, and Binary Mixtures in Fish Heptoma Cell Line PLHC-1. Environmental Toxicology and Chemistry 20: 149-159.	**83**
Liebsch M. (1997): Entwicklung eines neuen in vitro Tests auf dermale Phototoxizität mit einem Modell menschlicher Epidermis. ALTEX 14: 165-174.	**29**
Hernandez Y., Fischer R. (2007): Serum-free Culturing of Mammalian Cells – Adaptation to and Cryopreservation in Fully Defined Media. ALTEX 24: 110-116.	**29**
Rothen-Rutishauser et al. (2008): A Newly Developed In Vitro Model of the Human Epithelial Airway Barrier to Study the Toxic Potential of Nanoparticles ALTEX 25: 191-196	**47**

Sauer U. (2009): Animal and Non-Animal Experiments in Nanotechnology – the Results of a Critical Literature Survey. ALTEX 26: 109-134.	**18**
Tréz T. (2008): Instrumento Animal - o Uso Prejudicial de Animais no Ensino Superior. Canal 6.	**11**
Lenz et al. (2009): A dose-controlled system for air-liquid interface cell exposure and application to zinc oxide nanoparticles. Particle and Fibre Toxicology 6: 32	**126**
Stingl L., Voelkel, M., Lindl T. (2009): 20 years of Hypertension Research Using Genetically Modified Animals: No Clinically Promising Approaches in Sight. ALTEX 26: 41-51.	**3**
Brandenberger, C. et al. (2010): Quantitative evaluation of cellular uptake and trafficking of plain and polyethylene glycol-coated gold nanoparticles. SMALL, Volume 6 (15): 1669–1678	**182**
Brandenberger, C. et al. (2010). Intracellular imaging of nanoparticles: Is it an elemental mistake to believe what you see? Part Fibre Toxicol: 7, 15	**68**
Wick, P., Clift, M.J.D. et al. (2011). A Brief Summary of Carbon Nanotubes Science and Technology: A Health Safety Perspective. ChemSusChem 2011, 4, 905 – 911.	**36**
Andrea D. Lehmann, et al. (2011): An in vitro triple cell co-culture model with primary cells mimicking the human alveolar epithelial barrier: Eur J Pharm Biopharm. Apr;77(3):398-406	**85**
Müller, L., et al. (2011). Realistic Exposure Methods for Investigating the Interaction of Nanoparticles with the Lung at the Air-Liquid Interface In Vitro. Insciences J. 1 (1), 30-64	**35**
C. Brandenberger ,B. Rothen-Rutishause, et al. (2010). Effects and uptake of gold nanoparticles deposited at the air–liquid interface of a human epithelial airway model: Toxicology and Applied Pharmacology 242, 56–65	**140**
Navabi N., McGuckin M. A., Lindén S. K. (2013): Gastrointestinal Cell Lines Form Polarized Epithelia with an Adherent Mucus Layer when Cultured in Semi-Wet Interfaces with Mechanical Stimulation. Plos One 8: e68761.	**36**
Leach M. C. et al. (2011): Are We Looking in the Wrong Place? Implication for Behavioural-Based Pain Assessment in Rabbits (Oryctolagus cuniculi) and Beyond. Plos One 6: e13347.	**40**
Behrensdorf-Nicol H. A. et al. (2013): Binding and cleavage (BINACLE) assay for the functional in vitro detection of tetanus toxin: Applicability as alternative method for the safety testing of tetanus toxoids during vaccine production. Vaccine 31: 6247-6253.	**6**
Behrensdorf-Nicol H.A., Weisser K., Krämer B. (2015): "BINACLE" assay for in vitro detection of active tetanus neurotoxin in toxoids. ALTEX. 32:137-42.	**0**
Kloezen, W., et al. (2015): A *Madurella mycetomatis* Grain Model in *Galleria mellonella* Larvae. PLoS Negl Trop Dis. 2015 Jul 14;9(7):e0003926	5
Vullioud S., de Kaenel I., Schindler S. (2016): 3rupdate.ch: a new online educational tool for improved 3R literature search.	Not found
Publications: 28	**1'401**

ALTEX

With increasing acceptance and better understanding of the 3Rs principles increasingly catching on, there has been a strong development towards more and scientifically more valuable project applications, which is indicated in the rise of quality indexes (impact factors) of ALTEX, which publishes 3R relevant work [57].

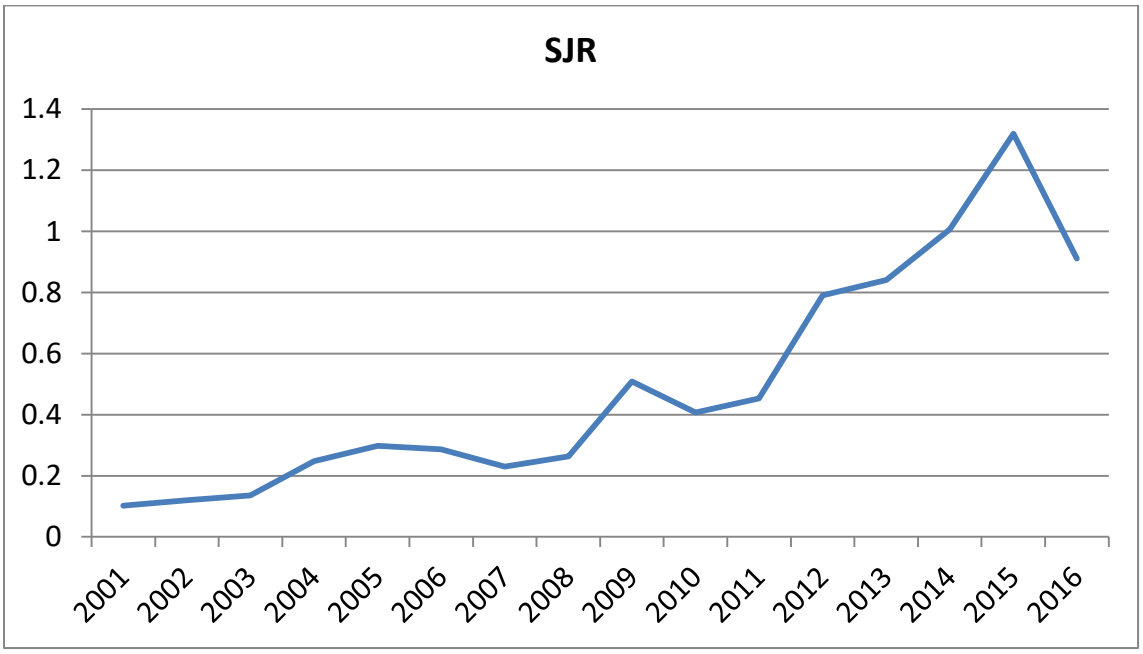

Fig. 2: Scientific Journal Ranking (SJR) of ALTEX

The SJR is a size-independent prestige indicator that ranks journals by their 'average prestige per article' [58].

It is based on the idea that 'all citations are not created equal'. SJR is a measure of scientific influence of journals that accounts for both the number of citations received by a journal and the importance or prestige of the journals where such citations come from. It measures the scientific influence of the average article in a journal, i.e. it expresses how central to the global scientific discussion an average article of the journal is.

The SJR measures both the number of citations received by an article as well as the importance of the journals citing it.

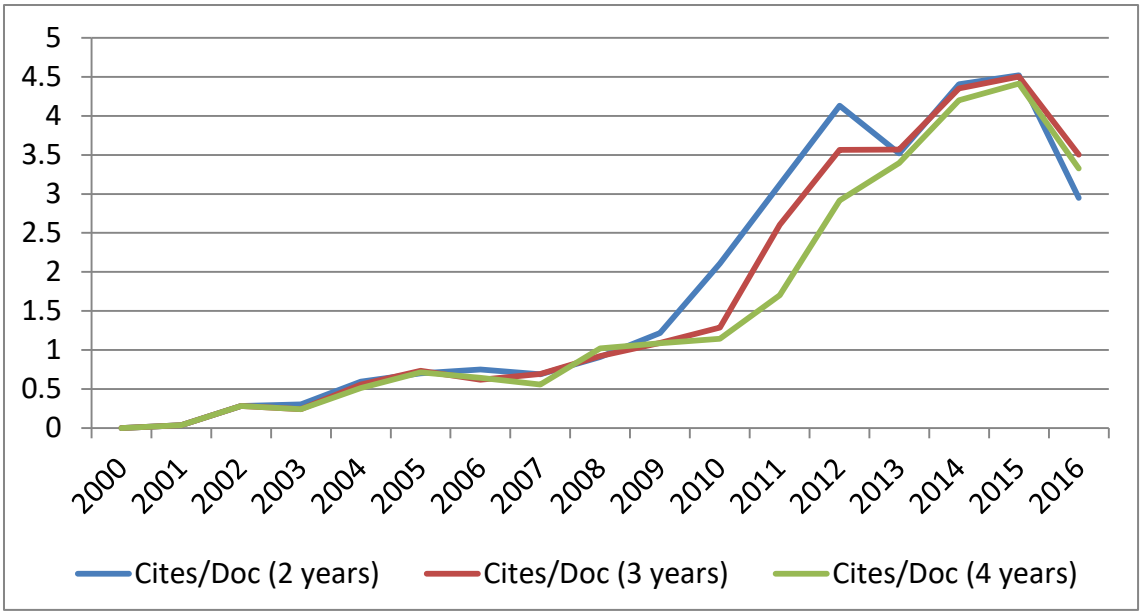

Fig. 3: Citations of ALTEX articles 2001-2016 according to SJR

This indicator counts the number of citations received by documents from a journal and divides them by the total number of documents published in that journal. The chart shows the evolution of the average number of times documents were published in a journal in the past two, three and four years have been cited in the current year. The two years' line is equivalent to journal impact factor ™ (Thomson Reuters) metric.

Journal self-citation is defined as the number of citation from a journal citing article to articles published by the same journal.

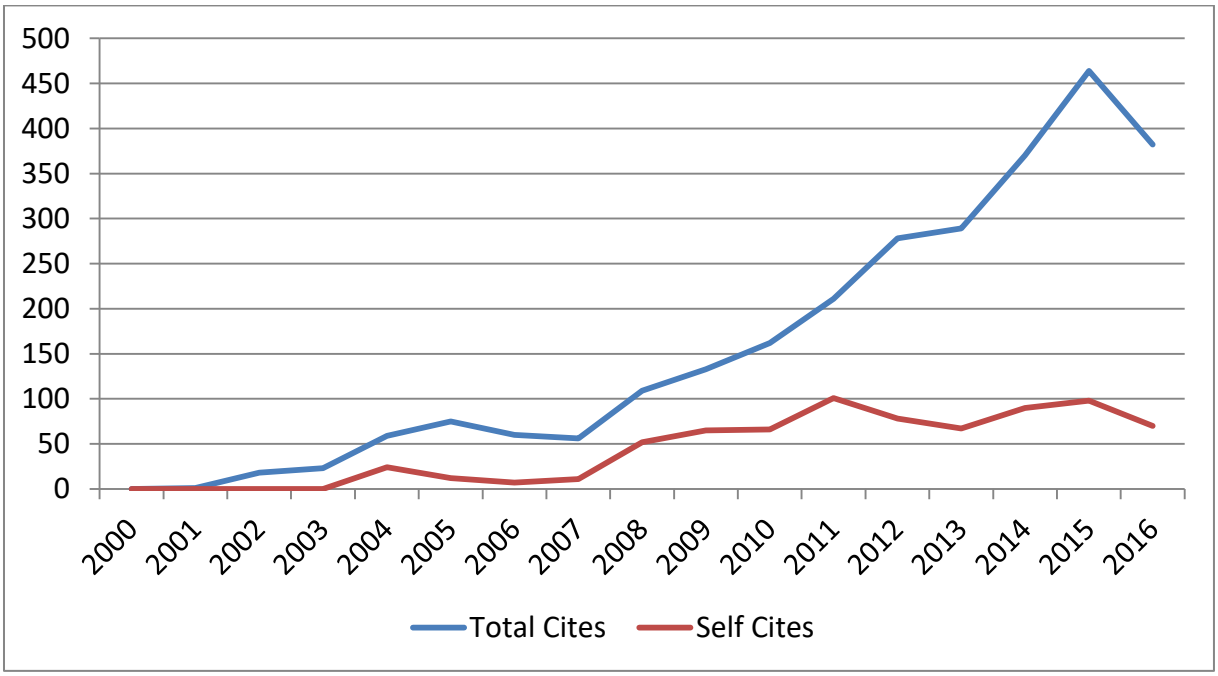

Fig. 4: Citations vs. self-citations from 2001 to 2016 according to SJR.

In 2012, the European Centre for the Validation of Alternative Methods (ECVAM) issued a guide on data retrieval principles for untrained users. In this, search terms for finding 3R-relevant publications are presented. Obviously, the Foundation so far has not insisted that the publications financed by it bear a clear declaration of the foundations' goals and intentions. For this, one has to keep in mind that the AfR was founded in 1976, a time when in vitro methods were in their very beginnings and animal-free testing was regarded (at best) as quixotic. It is understandable that in this context, applicants were not persuaded to give their papers a particular spin towards the 3Rs.

The rationale behind these facts is as follows: the AfR differs from "normal" funding organizations in several important aspects. Unlike others, the scientific success and a high scientific standard of the work is important, but not the only feature that is decisive for success or failure of a given project. As depicted later, the effects on (long-term) animal welfare, be it reduction of animal numbers or reduction of animal distress are at least equally important. For decades, this was not mirrored in the measurement of project outcome on a regular basis. With the increasing importance and recognition of the 3R concept, and researchers scanning the databases for publications that can help them implement or improve implementation of 3R in their work, this gap is starting to show. Neither are the necessary keywords for a successive search included nor does it from the context become apparent, that this work was funded by an organization with the explicit goal to advance alternatives to animals. This is expressly lamentable, and it remains to be seen whether there is a way to rectify this retrospectively, albeit definitely in the future.

4 Overall discussion and conclusions

4.1 Principles of evaluation

The AfR foundation has been existing since 1976; this is the first study to evaluate the activities, and especially, the impacts that the work has had. The evaluation covers the time period of 40 years, 1976-2016. As in former work e.g. by Gruber et al. [59] and Rusche et al. [60], the scientific value of the project work was not subject to the evaluation. Unlike the abovementioned study, this work tried to explicitly address the outcomes and impacts of supported projects.

Methodically, this study transcends former work in a way that it attempted to evaluate the actual impact on animal welfare. For this, it defined criteria as to the success (3R impact) of projects. Since the evaluation was not doable without the help of former project leaders, a special questionnaire was developed, the results of which are presented in this study.

With regards to project success, it has to be noted that in the case of 3R-relevant projects publication is by far not the only indicator. This distinguishes the work from the activities promoted e.g. by the Swiss National Science Fund. In several cases, a publication was not even the ultimate goal of the project. Furthermore, one has to consider that an impact on animal welfare takes time to develop, and is subjected to a variety of imponderabilities (e.g. validation or further development of a given approach), which enhance or change the potential to influence animal welfare. In this respect, a project can only be regarded as unsuccessful if there is not and probably never will be, an impact on animal protection and welfare.

As in the previous work by others, this study was hampered to great extent by lack of complete information. Especially with regards to projects performed before 1996, the return rate for the questionnaire was very low (it has to be mentioned that project leaders had already responded to a questionnaire in 2004, and some apologized, that they genuinely did not remember), citation rates in Scopus® cannot be done before 1996, (and especially for journals concerning themselves with the 3R concept and/or animal welfare only long after); and some publications before 1996 were not accessible as full texts. All the numbers provided in this study (number of publications, number of citations) are minimum numbers, comprising only those publications where it could be ascertained without any doubt that they carried an acknowledgement of the FFVFF or AfR.

The evaluation period ends in 2014. With regards to “late” projects, for several aspects, only projects that were completed in 2008 could be assessed. This holds true for the citation analysis and especially for the 3R impact, which requires time to develop, as is demonstrated in this study.

It is impossible to gather all effects of a given project. This holds especially true for exact animal numbers, which are impossible to determine in a way that can still be called scientific. The reasons for this are thoroughly discussed. Success was divided into scientific impact (publication and citations) and impact on animal welfare. Assessing this latter impact of 3R Research is indeed a difficult task. As Leist et al. stated in 2008: the effects tend to be underestimated. There are several reasons for that, and amongst the most important for Switzerland are the following:

- non-distressing methods (SD 0) are included into the statistics, resulting in apparently rising animal numbers;
- altered definitions of experimental animal: foetuses included, cephalopods, decapods, etc.;
- and, as could also be shown here, expansion of scientific research.

With regards to publications, the effects are absolutely certain to be underestimated according to the authors. For example, in vitro pretesting is not published in basic research, and therefore there exists a positive publication bias for in vivo studies. It is for example still commonplace that a single animal experiment with negative data can be published. The present status quo makes such a publication with alternative methods unthinkable. The analysis of keyword and contents performed in the assessment presented here supports this finding.

4.2 The 3R concept

The changes in attitudes towards the 3R concept that have taken place since 1987 are immeasurable. As we have seen in the responses to the questionnaire, the respondents reported a high rate of positive feedback for their work on improving the welfare of many experimental animals, a finding that is most encouraging. In this light, it would have been interesting to learn more from the "older projects" dating from the early years, to see whether there has been a measurable development. Unfortunately, 40 years proved simply too long to do a retrospective evaluation, since the return of these questionnaires was very low, and some of these applicants communicated that they simply (and quite understandably) do not remember.

Doing such a historical study is an entire enterprise in itself. Focusing only on the recent years, Switzerland has introduced the dignity of the animal into its Animal Welfare Ordinance in 2008 and into article 26 of the Animal Experimentation Ordinance, the EU has issued a Directive on the protection of laboratory animals that places huge emphasis on the 3R concept, and in the USA, the National Research Council NRC has stunned animal welfare with its appeal to apply a completely new paradigm in toxicity testing – away from animal testing.

All in all, the 3R concept is widely embraced and has opened up a line for widespread collaboration between animal welfare and researchers, leading away from confrontation and opening up the possibility to communicate and formulate common goals.

Still, animal welfare organizations (like the AfR) give replacement priority over reduction and refinement, with replacement (absolute replacement) and research without the use of animals being the ultimate goal. In contrast, the priorities of researchers appear to be reduction / refinement, both of which have been (and still are) implemented very effectively, and quite often go hand in hand with each other. Actually, one of the main sources of misunderstandings between animal welfare and research is the fact that the interpretation of priorities and significance of the different "Rs" varies between these two interest groups.

Refinement is immensely important, affecting a huge number of animals, and is usually quickly and effectively implemented. Unlike the other two, which concern themselves exclusively with the experimental procedure itself, refinement comprises handling, husbandry (e.g. feeding, lighting, housing), and transport as well as measures to relieve pain and

distress before, during and after the experimental procedure. As a consequence, refinement measures affect the entire lifespan of an experimental animal. It has been shown that refinement measures, like behavioural enrichment, do not disrupt data and do not affect reproducibility. To the contrary, the understanding that improvements in animal welfare lead to better science is now uniformly accepted in Switzerland.

4.3 Continuous rise in animal numbers at academic institutions

Reasons why alternatives to animal experiments (not speaking of refinement!) are not widely adopted in basic research are manifold. As Gruber and Hartung have stated in 2004, there are specific hurdles to the implementation of alternative methods in basic research: for one, the enormous specialisation in basic research, employing very individual setups [61]. A possible consequence of this is that the alternative method is only used in the project it was developed for, but not in other groups working in similar fields. In addition, the use and further development / optimization are often not published.

Tradition is another point to consider: unlike in industry with their routine testing, research in basic science builds upon the results of former projects. As a continuous process, research in basic science provides some answers, but raises other questions, answers to which are then sought in follow-up projects. Scientifically, it is perfectly logical to use the same methodologies in all these approaches in order to achieve continuity and comparability. A switch to in vitro methods at some point is therefore difficult since it might jeopardize the data for considerable time (if in vivo and in vitro are not comparable), and in addition it might be difficult to explain this change to the editor of the journal in which you wish to publish. A solution would be to run in vivo and in vitro in parallel for some considerable time in order to prove the comparability or to implement measurements and endpoints in vitro that can serve as reliable surrogates for the in vivo endpoints. This approach might however be limited by the resources of a laboratory in an academic institution.

As a consequence, industry – which is strictly regulated and not free in their choice of methods – has implemented replacement / refinement approaches to a much greater degree and much more efficiently and systematically than basic research – which is free in their choice of methods. Although alternative approaches are successfully developed and implemented in the respective labs, the widespread distribution appears to be hindered by a number of factors. According to Ibrahim 2011 [62], a major inherent deficiency of the Three R's is that they were not designed with new and emerging technologies in mind. The author speculates that lack of implementation of in vitro methods may be due to: a) comfort with the traditional animal model, b) the vested interests of institutional players in animal research, c) fear of deviating from the status quo and the legal liability that may accompany that decision, and d) irrational insistence on high-fidelity models. In addition, the 3Rs stop short of questioning the overall purpose of an experiment on the grounds that it is trivial, unnecessary, or of questionable utility (or of an entire field of research, for that matter, personal comment by the author). On the contrary, they are designed to accept any experimentation that takes the 3Rs into consideration.

In the US, the Animal Welfare Act states that the International Animal Care and Use Committees (IACUCs) cannot challenge the "design, performance, or conduct of actual research".

For academic researchers, the pressure to publish may be a factor, as far more papers can be published in a given time period if animal experiments are used.

Potential replacements are evaluated in comparison to the traditional in vivo experiment. This is problematic on multiple levels:

The animal experiment may not provide reliable data. This problem has been stressed by many. In fact, it leads to the paradox that the worse the in vivo experiment is (the worse the data), the more difficult a replacement is, because you do not know which data are the "correct" ones. Additionally, it creates the problem that 3R is always lagging behind – it is, as a consequence, impossible to follow the rapid creation of GMOs and prevent it with the development and implementation of 3R methods. Much rather, a paradigm change is required to take place, moving away from in vivo and towards the use of cell lines and siRNA, for example, or genetically modified embryonic stem cells (or induced pluripotent stem cells).

4.4 The situation in Switzerland

On 9th of September 2000, the European Science Foundation ESF published a codex in which national societies are explicitly called on to endorse the 3R principles and include them into their funding rules. In Switzerland, funding of projects by the Nationalfonds (the Swiss Research Council similar to "Deutsche Forschungsgemeinschaft") requires adherence of the scientists to the ethical principles and guidelines first established in 1994.

Paragraph 4.6 of these guidelines states that in case of unbearable suffering of animals the experiments must be declined. In addition, animal experiments, which do not concur with these guidelines may not be exported to other countries.

Article 22 of the Swiss Animal Welfare Act states:

1. The Federal Government operates and supports scientific research of relevance to animal welfare.

2. In collaboration with universities and industry, the Federal Government in particular promotes the development, accreditation and application of methods which replace animal experiments, which enable fewer animals to be used, or which result in less strain for the animals. In particular, it promotes research projects aimed at eliminating pain, suffering or anxiety in surgical procedures as defined in Article 16.

The trend is clearly going in the opposite direction: the discrepancy between funding is notable: in 2009, the Swiss National Science Fund funded altogether 521 projects using animals, at a cost of 76 million Swiss Francs. In contrast, the Swiss taxpayer finances the 3R Research Foundation as the only state-funded organisation with around 450'000 Swiss Francs each year (but in 2016: 0 CHF). The power relationships are clear. This demonstrates that the value accorded to alternatives to animals, despite all speeches to the contrary, is still not fully established, while the worth of conventional science remains unquestioned.

4.5 Scientific impact

This study finds that the output of the FFVFF and later AfR with regards to publications is very satisfying. The publications in journals that specialize in animal welfare or the 3R-concept has decreased, and stopped entirely in 2005, indicating that work dedicated to replace / reduce / refine animal use is no longer an isolated topic, but has arrived in the mainstream. All the more important, though, that retrievability is ensured, which can be achieved by providing the paper with an appropriate keyword as suggested e.g. by the EURL-ECVAM Search Guide. As this study finds, in an overwhelming majority of publications, this is not the case. With regards to contents, papers frequently do not declare that the purpose of the work was the development or improvement of a 3R relevant method.

As was already stated by Gruber and Hartung in 2004, one of the major impediments to adoption and implementation of replacement and reduction approaches is a lack of information. The difficulties of doing an effective search of existing alternative methods has been widely recognized in the recent years. In consequence, e.g. authorizing bodies usually have no overview over existing alternative methods, because they have no access to the full text publications. In addition, even if they know of one they cannot necessarily judge the applicability of this method for this particular scientific approach. This is the underlying reason why the Swiss Animal Welfare Ordinance firmly places the information whether there would be an alternative method available, in the hands of the applicant. In conclusion, if members of the approving authorities cannot read 3R-relevant publications full-text, they are at a distinct disadvantage.

However, mere citations and the respective descriptions in the published articles are usually too brief to allow the adoption of a method. Furthermore, it is often difficult to trace the series of amendments of a method over time when only the original description is found.

4.6 Transparency and open access

The call for more transparency does not come out of nothing. A better understanding of research appears to be vital. The issue of animal experimentation and the funding and use of 3R-relevant methods must be a subject of informed public discourse. With regards to scientific publications, the current situation needs to be improved. Currently, (in the case of publicly funded research), a private person has to pay three times: once for the research generating the data, once for the universities licenses, and, if he or she want to read a paper him/herself, again to the journal for being permitted to download it.

An endorsement of Open Access is therefore highly desirable, as SNSF has recently announced. For animal experimentation, the ARRIVE guidelines have currently been adopted by more than 300 journals, including the PLOS family.

4.7 Animal welfare impact

This study found that the current documentation does not enable the AfR to determine an animal welfare impact. A decrease in severity degree of a given procedure is measurable and quantifiable, while the potential improvement of husbandry conditions is not. However, as has been shown in the study by Lindl et al. in 2001 [63], examining 51 project proposals at

a German University, 60% of the scientists had underestimated the level of suffering of the animals.

Animal experiments seem to be overestimated with regards to their relevance. Among the reasons are publication bias, publication of subgroups, or the use of inbred strains of mice (which has led to a reduction in animal numbers) with reduced variation in results due to extreme genetic homogeneity, but more than questionable relevance for an outbred population of human beings. The reproducibility crisis is yet another issue. Whether it applies less to in vitro methods than animal experimentation needs to be investigated.

With regards to replacement, two developments can be described. In the past decades, the low-hanging fruits have been picked. That is to say, those animal experiments that could easily and quickly be substituted by an in vitro method have been identified and replaced. This “1:1 replacement” is now (certainly with exceptions) a thing of the past. The animal experiments that are left are far more complex, with approaches that cannot possibly be mirrored in one single in vitro assay. The solution that has been proposed are so-called integrated testing strategies (ITS) that comprise a battery of in vitro tests (or computer simulations) that are either run one after the other or in different combination. Currently, a lot of work is being done in this field, with a large potential for replacing animals.

Is there also a difference in motivation between the two basic areas of science? There might be. Industry is cost-oriented, and as we have seen above, animal housing is a big factor (that is not including the cost of the animals themselves, such as breeding, transport, salaries of caretakers, animal welfare officers, veterinarians etc.). Industry is oriented on the results, too. They have to generate data that are relevant for judging e.g. a given risk for a human consumer. If they (and the regulatory authorities) can be convinced that an in vitro method does the job just as well, they will adopt it and terminate animal use. So, industry is very interested in replacing the costly in vivo approach, and they are able to quickly do so because of short and efficient decision-making processes.

In contrast, cost is (or rather, until recently has been) not much of an issue in academic institutions, the expenses for animal housing and husbandry being carried by the taxpayer. Secondly, science is much more individualistic and diverse. It is virtually impossible to offer a 3R method that is 100% suitable for any given approach in academic research. On the other hand, one might argue that there is a lot more flexibility in project planning, and, unlike in regulatory testing, the free choice to use whatever method is deemed suitable (if veterinary authorities permit it, that is). Still, it is an assumption that researchers in this field are interested in reduction and refinement, but basically have an interest to stay with the animal experiment. This is supported by a third finding, concerning the journals in charge of accepting publications. In vivo data are actually demanded by the editors, because they are regarded as the gold standard and as more relevant than in vitro data. Since the entire goal of the enterprise at the universities is the successful publication in a high impact journal, this constitutes a very major obstacle to the use of in vitro approaches. In this field, these can only play a supporting role, with the data from the animal having the last word.

So, as we have seen when looking at the distribution between applicants from academic institutions and those from industry, it becomes obvious that the AfR is predominantly stuck with the more complex and diverse field of basic research. Nevertheless, given the complexity of the task and the generous funding, it does seem a little unfair to blame the Foundation for lack of success.

But are the numbers depicted in the statistic above the whole truth? If the number of projects is on the rise, certainly a rise in animal numbers is to be expected, irrespective of success or failure of the 3R concept. If indeed, we take a glance at the developments in basic academic science, the picture changes entirely: the number of approvals has risen considerably, but at the same time, the number of animals per approval has fallen. Had it remained the same since 2005, there would have been a very considerable rise instead of a decline in animal numbers. The following table (Tab. 2) and figure (Fig. 5) calculate these hypothetical animal numbers.

Tab. 2: Method of calculating an average of animal numbers per approval

Year	Average animal numbers per approval	Average (rounded) of 2001-2005
2001	335	
2002	379	
2003	344	
2004	360	
2005	374	**358**

The number 358 forms the basis for the next graph (Fig. 5), which looks at what would have happened had the reduction efforts not been so successful. As a comparison, the animal numbers as they were actually observed in reality are shown. As can be seen, without reduction, animal numbers would have surpassed 1 million in 2010.

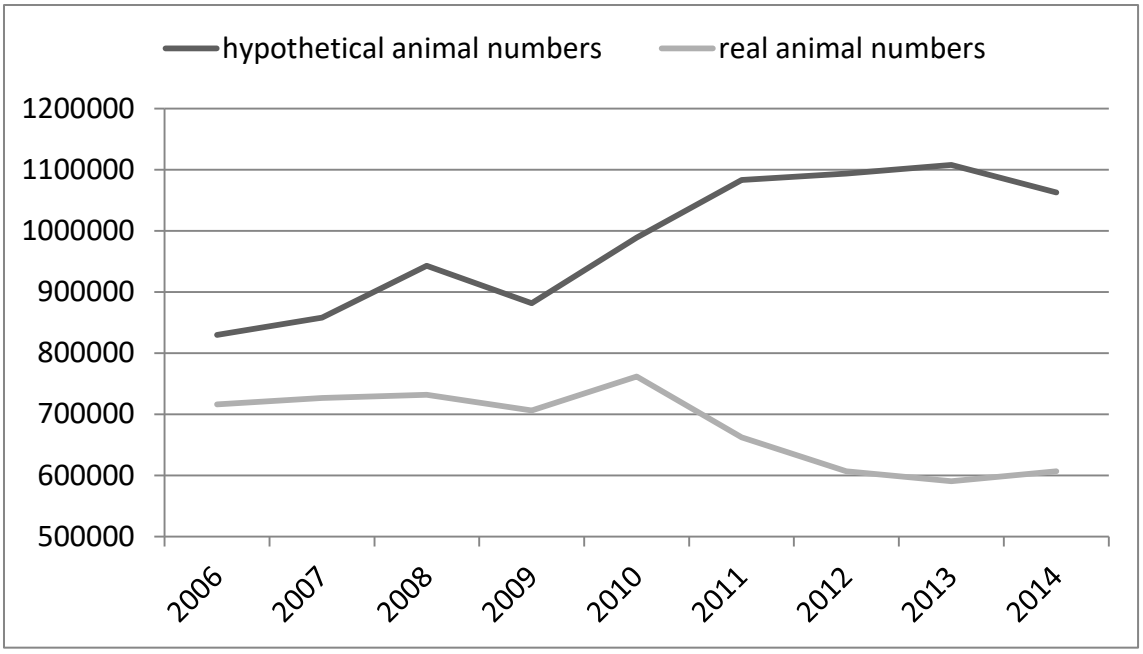

Fig 5. Hypothetical and real numbers of animals per year in Switzerland (please note that the graph´s axis starts at 500´000).

It is always difficult (and in fact unscientific) to speculate. It is impossible to know how much of this success can be attributed to the work of the AfR. Equally impossible as it would be to count the saved or affected experimental animals, is the assessment of intangible assets, such as information of the 3Rs, motivating researchers to file an application and enthuse them for the topic, and possibly much more. But we can state confidently that in the years 1976-2016, the AfR has had successes – its work has had a concrete impact on animal numbers. And although it is not possible to trace all the methods through all adopting working groups and retrieve the animal numbers before and after, it is safe to say that the AfR has saved numerous animals and it has affected their welfare in a profound manner. Non-measurable things like raising awareness levels, educating and alerting the new generations of researchers, winning scientists over to embrace the 3R concept, adopt and propagate it with sincerity and enthusiasm, cannot be underestimated.

Is funding the 3R concept different from mainstream science? It is indeed. It turns out that one of the fundamental and long-lasting errors has been to make the funding and support of such projects an exact copy of conventional funding. In mainstream science, a project proposal is evaluated for different criteria (e.g. plausibility, status of the project leader, probability that the proposed approach will be successful), and after conclusion, the funder usually demands a publication carrying an acknowledgement. With the publication step, the work is regarded as having been made widely accessible and available, and therefore successful.

In exactly the same vein, the foundation Animalfree Research has approved and funded projects. An additional criterion for acceptance, is the potential for the developed method to carry a profound and long-lasting impact on welfare improvements. But then, in an exact parallel, the success of a given project was measured by publications.

Basically, this alone is not enough. The publications must be retrievable and readable for everyone; it therefore requires the proper keywords and the endorsement of open access. In

addition, publications do not fully reveal the impact on animal welfare. A new method is published with its scientific merits, but hardly ever is it mentioned that it has the potential to affect animal welfare. Especially in basic research (where animal numbers are on the rise), it proved to be absolutely impossible to retrieve these papers in a realistically doable search. This issue is reflected in the generation of databanks over databanks, all designed to provide retrieval of the necessary information. The publication of the ECVAM Research Guide on Alternative Methods is a much-awaited guide through the impenetrable forest, but despite all efforts, in a given research area, it is not possible to retrieve the full spectrum of publications of a given alternative that might be relevant to the researcher.

In the future, all 3R organizations have to stress in their contracts and arrangements the necessity for the researchers to include the necessary keywords. Still, the fear that a lot of former work is more or less “lost” cannot be dismissed.

A major task in convincing researchers to switch to animal-free alternatives would be the facilitation of publication with in vitro methods. Public and political pressure might enhance the willingness of editors to accept such papers. At this point, nevertheless, it is unclear how changes could be brought about in this area and if so, what role the AfR could assume in this task.

Recently, the severe issue of underpowered studies has been raised. As Button et al. 2013 [64] have convincingly shown for the area of neuroscience, this is capable of impeding the progress and, worse, ruining the reputation of an entire research field by producing and publishing coincidental data as results. Here, the honourable efforts of approving authorities and researchers alike, to allow for and use as few animals as possible, have obviously turned out to be counterproductive, since the goal of the 3R principle is explicitly good science with fewer animals.

Since the solution cannot possibly be to increase animal numbers again, in vitro data must increasingly supplement, or ideally replace the animal experiment. Supporting this, in the 2013 questionnaire, a great majority claimed as one of the major assets: “More and better data”.

It is not the aim of the 3R concept to ruin science and produce irrelevant data; data that are not reproducible create sheer havoc, also with regards to animal welfare, when researchers try to recapitulate the experiments, and all the worse since these failures aren’t published. On the other hand, no one could wish that the number of animals used for experiments should increase. The above experiences may be the way out: as far as at all possible, replace or supplement animal data. As Gruber and Hartung state: “With in vitro methods, replications are usually not an issue”.

4.8 Criteria and caveats for assessing the impact of 3R-relevant work

A recommendation to existing and upcoming 3R Research Centres:

Impact on animal welfare

To understand the impact on animal welfare, the following criteria were evaluated:

- Scientific success
- Success with regards to the 3Rs:
    - Were animal numbers reduced?
    - Was animal welfare improved?
        - Reduction in severity degrees
        - Improvement of animal welfare through animal housing, handling and husbandry
    - How long was the method in use in the former applicant´s laboratory?
    - Was the method adopted by other groups? Permanently? Was it adapted, improved?
- "Soft successes", such as
    - Increased work satisfaction
    - Improved science through more and better data
    - improved motivation of employees

Further success criteria on the impact on animal welfare are the adoption of the method by other working groups, long-term implementation at least in the developing laboratory, and adoption into national or even international regulatory frameworks.

5 Abbreviations

3RRF 3R Research Foundation

AEDs Anti-Epileptic Drugs

AfR Animalfree Research

ALICE-CLOUD Air-Liquid Interface Cell Exposure System

ALTEX Alternatives to Animal Experiments

ASCCT American Society for Cellular and Computational Toxicology

ATLA Alternatives to Laboratory Animals

BfR Bundesinstitut für Risikobewertung

BINACLE Binding and Cleavage Assay

CAAT Centre for Alternatives to Animal Testing

ECVAM European Centre for the Validation of Alternative Methods

EDQM European Directorate for the Quality of Medicines and Healthcare

EEG Electroencephalography

ERGATT European Research Group for Alternatives to Animal Testings

ESF European Science Foundation

ETHZ Swiss Federal Institute of Technology in Zurich

EU European Union

EUSAAT European Society for Alternatives to Animal Testings

FBS Foetal Bovine Serum

FFVFF Fonds für Versuchstierfreie Forschung

GLP Good Laboratory Practice

GMO Genetically Modified Animals

HIFU High-Intensity Focused Ultrasound

IACUCs International Animal Care and Use Committees

IBM International Business Machines

InterNICHE International Network for Humane Education

ITS Integrated Testing Strategies

IV Intra Vascular

LD_{50} Lethal Dose 50

LPS Lipopolysaccharide

mA Milliampere

MEGAT Mitteleuropäische Gesellschaft für Alternativen zu Tierversuchen

MES-test Maximal Electroshock Induced Test

PLHC-1 Permanent Hepatoma-Cell-Line

PTZ Pentylenetetrazole

RTgill-WT Rainbow Trout Gill-Waterloo1 Permanent Cell Line

sec second(s)

SCC Squamous Cell Carcinoma

SIAT Schweizerisches Institut für Alternativen zu Tierexperimenten

siRNA Small Interfering RNA

SJR Scientific Journal Ranking

6 Summary

This report was commissioned by the Animalfree Research (AfR) foundation to follow-up on projects which have been either partly or fully financed by the AfR over the last 40 years. It was assessed what impact each project had on future research up to the present day – with the main focus on the outcome of the project in supporting the reduction, replacement and refinement of the use of animals in research. Methods of analysis included researching the internet, reviewing old documents and interviewing former project leaders or project members. This report covers 20 projects in total. In regards to the publication output, the results are very satisfying. The publications in journals that specialize in animal welfare or the 3R-concept has decreased, and stopped entirely in 2005, indicating that work dedicated to replace / reduce / refine animal use is no longer an isolated topic, but has arrived in the mainstream. While the current documentation unfortunately does not enable determination of the exact impact on animal welfare, it was very likely significant – considering how often the studies supported by the AfR have been cited. With animal numbers on the rise in basic research, the majority of which is performed in academic institutions, it is vital that the AfR continues its efforts and maintains a focus on the 3R area.

7 Acknowledgements

We are, unfortunately not able to provide a comprehensive list of everyone who has contributed, we would like to use the opportunity to thank everybody who contributed to the success of the Animalfree Research foundation. In particular, we are grateful to our founders!

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We would furthermore like to express our heartfelt gratitude to Monica Gaiffi for writing, and to Dr. Miriam Zemanova for finalizing this report.

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9 Appendices

9.1 Appendix I: General form of a funding contract

Contract on the allocation and use of research funds by the foundation Animalfree Research

Between the foundation Animalfree Research
(sponsor)
and
……..
(recipient)
represented by
……..
the following contract is concluded:
The project will be initiated and conducted by **……..** as the Project leader at the **……...**

Art. 1 Object of funding

The foundation supports the following projects *as described by the recipient in the attached project proposal.*
Project 1: **……..**
Project 2: **……..**

Art. 2 Duties of the recipient

a) The recipient employs the grant for the optimum use of the above project and allocates the funds in the manner specified in the grant application. In case of a change of allocation, the sponsor has to be notified in advance.
b) If the project plan as submitted to the foundation undergoes significant changes regarding its goals with ensuing loss of 3R relevance, or if the project is cancelled before time, the sponsor reserves the right to reclaim the fund (entirely or partially). The sponsor can abstain from this reclaim if the results generated so far are suitable for publication and show relevance for the replacement, reduction or refinement of animal experimentation.
c) In all publications relating to the funded project part, the foundation has to be acknowledged as sponsor, using its full name “Animalfree Research” (condition).
d) The sponsor is notified immediately if the project is cancelled or at serious risk

of not achieving its aims as depicted in the project plan.

Art. 3: Start of the funded project part and duration

The project starts on XX. XX. 20XX and ends on XX. XX. 20XX.
By XX 20XX the recipient submits a short interims report to the sponsor.
Reports and publications are then submitted as specified in the grant application (milestones).

Art. 4 Duties of the sponsor

Animalfree Research funds the project with a one-time donation of
…….. Swiss Francs.
…….. Swiss Francs in XX 20XX.
…….. Swiss Francs after receipt and approval of the short interims report in XX 20XX.
It is left to the foundation's discretion to grant further support for the **……..**, for associated other projects, travel expenses and/or congress fees.

Art. 5 Liability of the sponsor

The foundation Animalfree Research provides financial support to the project, but is neither its initiator nor responsible for its proper conduct. Especially, it is not responsible or liable for third party damages. These are the accountability of the project leader.

Art. 6 Information

The sponsor is entitled to request information on the project at any given time. The reports according to Article 3 require approval of the sponsor. The approval is to be regarded as granted if within 2 months after receipt of the report no objections have been raised by the sponsor.

Art. 7 Dissemination of results

Both parties endeavour to communicate the results of the research and their significance for the replacement/reduction of experimental animals to scientists, researchers and the general public and support each other in doing so.

Art. 8 Further agreements

1) Changes of and additions to this contract require written form.
2) The contract becomes effective once it is signed by both parties

9.2 Appendix II: Regulation for awarding research grants

Regulation for awarding research grants

Foundation Animalfree Research

24th of June 2014

(based on Art 9. 3 of the foundation's deed, 16th April 2008)

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I. General terms

Art 1 Purpose

This regulation serves to provide a basis for all operating procedures and decision-making processes related to applications for research grants handed to the foundation.

Art 2 Relation to the deed of foundation

The regulation specifies the foundation's deed. In the case of ambiguities or contradictions the deed overrules the regulation. Additions to the deed by the regulations are acceptable as long as they don't alter the purpose of the foundation.

Art 3 Time frames for funding

A grant cannot be awarded for a period of more than two years (with an option for prolonga- tion of 1 year). For longer-term grants see Art 5.

II. Conditions for funding

Art 4 Acceptable topics

In addition to the specifications of the deed (Art 2. 2) the following topics are accorded pref- erential sponsorship by the foundation Animalfree Research:

a) Advancement of alternative methodes for the complete or partial replacement of ani- mal experiments in terms of the 3R: development, validation, optimization, publica- tion, acceptance, implementation.

b) Dialogue with science and the public as well as commitment to the changes in the le- gal framework regarding the protection of experimental animals

Art 5 Regular (institutionalized) funding

[1] Particular events and activities serving the reduction and replacement of animal experi- ments can be supported for more than three years (e.g. World Congresses on Animal Use and its Alternatives, Linz Congresses).

[2] The support of congresses should allow, whenever possible, to play a steering role on the program design: e.g. by sponsoring particular sessions or by taking over travel expenses for scientists whose attendance is considered valuable.

[3] The decision which grants can be awarded for longer periods of time is determined by considerations concerning science, animal welfare politics and legal practice.

Art 6 Topics excluded from funding

[1] No animal experiments are financed by means of the foundation.

[2] Explicitly excluded from sponsorship are:

a) projects expected to have little or no impact on replacement and/or reduction of animal experiments.

b) scientific projects with no perspective to be implemented and used as an alternative method.

c) projects which disregard the animal's dignity.

Art. 7 Properties of Publications

[1]Publications deriving from a project supported by Animalfree Research must carry at least one of the following keywords:

„animal use alternatives“

„animal testing alternatives”

in order to facilitate their retrievability in a data bank search:

[2]Open access: The foundation aims at making publications resulting from funded projects accessible to all interested parties and therefore explicitly supports the “Gold Road of Open Access” (direct publication in an Open Access Journal of scientifically acknowledged quali- ty). If the costs for publishing in such a journal can verifiably not be carried by the author or his/her institution, an application for an additional maximum funding sum of 1’500 Swiss Francs can be submitted to Animalfree Research within the duration of the project. The de- cision on granting the additional sum is up to the foundation.

III. Application and evaluation procedures

Art 8 Application procedure

[1] All applications for sponsorship are to be directed to the foundation's office. The office con- firms receipt of the application within 2 weeks. The applications are treated as confidential.

[2] For applications, use exclusively the submission form on www.animalfree-research.org.

[3] The office audits the applications with a view to compliance to the rules, professional quali- ty, relevance to animal welfare, and budget compatibility.

[4] The foundation reserves its right to clarify the resources for executing a project on location, if necessary, and to pass the project application on to an external referee for his/her opinion. The applicant agrees to this condition through the submission of the application.

[5] As a rule, applications are being decided on within three months.

[6] Members of the steering committee, who apply for funding on their own or on an employer's behalf, must not be treated preferentially.

Art 9 Contract

[1] In case of approval of the project a sponsorship contract in written form is concluded be- tween the foundation and the applicant.

[2] The contract determines the frame of the granted support.

Art 10 Applicant's obligations

[1] Applicants are required:

a. to use the grant received for the purposes of the approved research project (and to present regular accounts).

b. to inform the office in good time if any funds already allotted are not likely to be needed.

c. to deliver the interim and final reports within the time limits decreed in the con- tract.

d. to inform the office of any patent applied for in relation to work carried out as part of the research project funded by the foundation. If a patent is registered and economically exploited, the Foundation is entitled to reclaim grants full or in part. In this case the Steering Board decides on the amount to be paid to the Founda- tion on an individual basis.

e. to submit the scientific results of their research project to an appropriate organ for publication, simultaneously submitting a copy to the foundation. All publications have to mention the support by the foundation.

f. hand in a short version suitable for the lay public.

[2] The foundation reserves the rights to use the results of the research project in a suitable manner in order to present its work to the public.

IV. Additional terms

Art 11 Modifications of the regulation

[1] Changes of the regulation require the approval of at least two thirds of the steering board members.

[2] The modified regulation is initialled by the President on each page and dated at the docu-
ment's end and signed as well as filed as an original with the foundation's records.

[3] Each member of the steering committee as well as the office and the supervising authorities
(Art 12. 4 in the foundation's deed) receive a complete copy of the modified regulation for their information.

Art. 12 Furtherance

This regulation enters into force with its approval by the steering committee. The original, dated and signed by the Co-presidents is filed among the foundation's records

Bern, 24th of June 2014

(Signatures)

9.3 Appendix III: Suggestion for a questionnaire for evaluating impact

The animal experiment		
	Did the developed method have actual and recognizable effects on reduction, replacement and/or refinement? (if the project had several 3R aspects, please feel free to mark more than one box)	Yes: ☐ Replacement ☐ Reduction ☐ Refinement:reduction of severity d ☐ 1 severity degree ☐ 2 severity degrees ☐ 3 severity degrees ☐ No
	All in all, how many animals were affected by the project in your own laboratory?	☐ according to existing documentatior ☐ according to estimates

	What area of research is affected by the method?	☐ Basic research/applied sciences ☐ Regulatory/QC ☐ R+D ☐ Diagnostics ☐ Validation ☐ Others: .....
	Which animal species was/were affected ?	
	What was the severity degree before the project/of the original animal animal experiment?	
	If possible, please give a **short** description of the critical aspect of the procedure performed on the animals that determined the severity degree (additional space at the end of the document)	

The project	Were your aims accomplished?	☐ Yes ☐ No ☐ In part
	Does the model fulfill your expectations regarding its scientific value?	☐ Yes ☐ No ☐ In part
	Does it fulfill your expectations with regards to impact on 3R?	☐ Yes ☐ No ☐ In part
	Was the method adopted by other working groups?	☐ Yes ☐ No
	How many?	
	Were there failed attempts to establish the method in other laboratories?	☐ Yes ☐ No

	In your lab, is the method still in use today?	☐ Yes ☐ No
	If yes, was it further adapted/optimized?	☐ Yes ☐ No
	If no, how long was it in existence?	☐ less than one year ☐ 1-3 years ☐ 3-5 years ☐ more than five years
	If the method is no longer used in your own lab, what were the reasons to discontinue its use?	☐ Back to animal experiment ☐ Different in vitro method ☐ Experiments no longer performed ☐ Others ……
	Are/were there SOPs or other standardized procedures concerning the use of the method?	☐ Yes which?…… ☐ No

9.4 Appendix IV: The visibility of the 3R concept

9.4.1 Approaches and methods

The basis for this evaluation is formed by multiple questionnaires which were handed out to the participants of the so-called Module 1, an obligatory course, which has to be completed by anyone who is going to work with laboratory animals in Switzerland. It is FELASA accredited and comprises lectures on handling, simple procedures, legislation, transgenic animals, and last but not least the 3R concept. It addresses students, e.g. PhD and masters, which typically constitute more than half of the audience.

9.4.2 Knowledge of the 3R concept

An interesting issue here might be to investigate the knowledge of the 3R concept among young scientists, i.e. people who have been educated in the natural sciences, very frequently biology and veterinary science. For evaluating the knowledge of the 3R principles, a small questionnaire was handed to the participants of the Module 1, starting in June 2013. The courses are taking place in English, and once or twice a year, depending on demand, also in German.

The question on the 3Rs was simply: "Do you know the principle of the 3Rs?". There were no further details required.

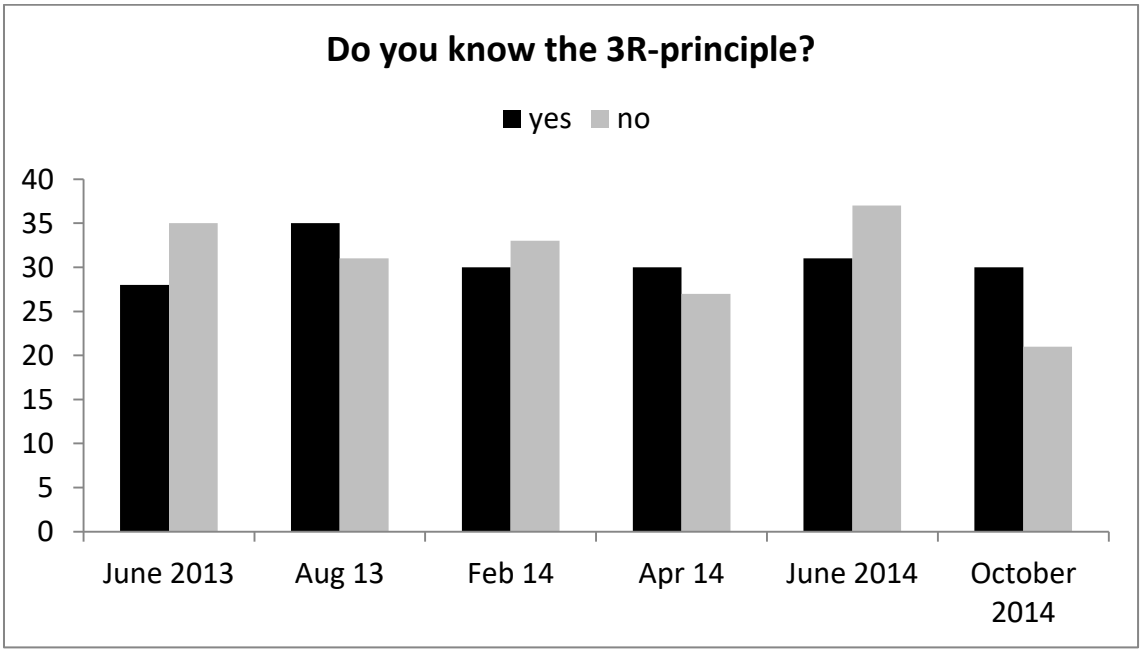

Fig. 6: Responses of participants of English-speaking Module 1 to their knowledge of the 3Rs principle, 2013 and 2014.

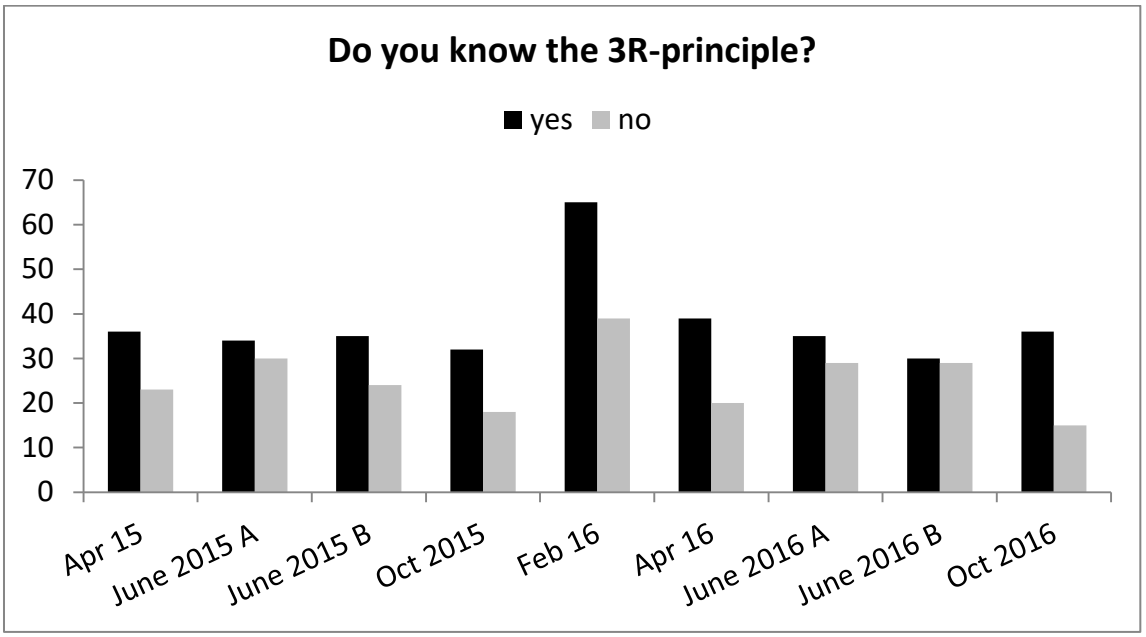

Fig. 7: Responses of participants of English-speaking Module 1 to their knowledge of the 3Rs principle, 2015 and 2016.

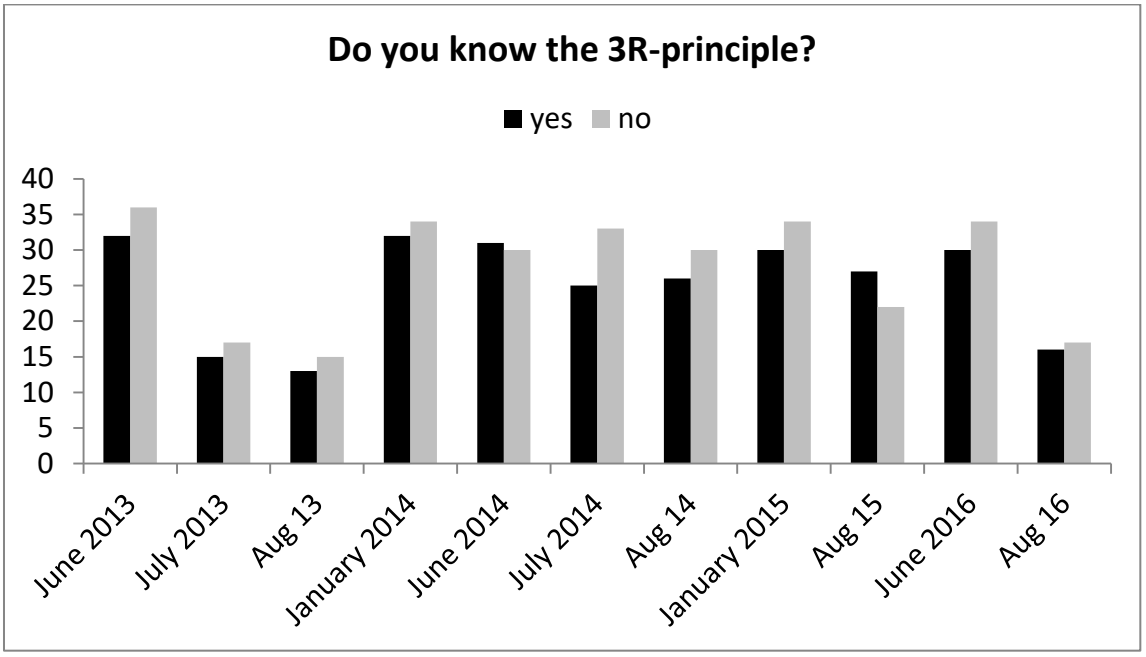

Fig. 8: Responses of participants of German-speaking Module 1 to their knowledge of the 3Rs principle, 2013- 2016.

In the German-speaking courses, with two exceptions, a slight but stable majority of participants have never heard of the 3R principle. This is all the more astounding since they have had a long lasting education that has (obviously) enabled them to principally perform animal experiments. Furthermore, it is a prerequisite that only those people may attend the course if they actually intend to start performing in vivo experiments in the near future. They are therefore aware of these future activities and might be expected to be already alerted to the difficulties of these tasks, and have acquired some information on methods and

possibilities to reduce and refine animal use. In the English-speaking courses, there appears to be an improvement (as it is) in the years 2015/16 when compared to earlier years. It is not clear how the change came about, it is nevertheless quite unspectacular. The basic findings hold true for both the English and the German participants.

9.5 Appendix V: Recommendations for future activities

With animal numbers on the rise in basic research, the majority of which is performed in academic institutions, it is vital that the AfR continues its efforts and maintains a focus on that area. With a background of the closing of 3RRF, at the time of writing, it is vital to not discourage researchers that are interested in improving the animal´s welfare in their particular field of expertise.

In a similar vein, it is recommended to maintain the broad bandwidth of the Foundation´s activities that comprise project funding, information of the public, political activities and education/counselling. Furthermore, the “open-topic” policy should be continued, where no calls for particular topics are issued. That way, a broad spectrum of applications is guaranteed to be submitted, making it possible to pick the most relevant and promising ones and perpetuate the current high quality of project selection in the future.

9.5.1 Documentation

It is encouraged to extend the already existing one (e.g. interim reports, final reports, email correspondence) with further regular questionnaires in order to facilitate further evaluations and provide the Foundation with the opportunity to react quickly and flexibly to changes.

Specifically, these are:

- A questionnaire that is being filled out by the project leader immediately after conclusion of the project. This information concerns itself with visibility and perception of the Foundation itself.
- A questionnaire that is sent out 3 years after conclusion of the project, which asks for the progress of a given method.

9.5.2 Publications

It is strongly suggested to make the use of appropriate keywords obligatory. The ECVAM Search Guide proposes “animal use alternatives” and “animal testing alternatives”. Furthermore, it is equally encouraged to promote open access publications, as is already endorsed by the SNSF. It has to be discussed whether AfR offers a financial compensation to authors who are interested in publishing in an open access journal, but cannot afford to. For details, it is advised to contact the network of the Swiss librarians that provide assistance in performing a search for the 3R relevant methods.

9.5.3 Visibility

As seen above, the 3R concept is not as well-known as it should. Therefore, it is suggested that AfR attend conferences, give talks or prepare posters, and write letters or short reviews for events and journals that are not predominantly concerned with the 3R concept. Also project holders should be encouraged to do so. One might consider funding the travel expenses to well-known conferences, e.g. the World Congress of Alternative Methods.

9.5.4 Implementation

It is suggested to arrange for a regular annual meeting among the network of the animal welfare officers, the AfR, and, if feasible, project leaders to present concluded projects, discuss the features and limitations of the developed methods and establish a personal contact between the persons concerned. The idea is to encourage AWOs to contact the project leaders personally if they feel that a given method might be applicable in one of the working groups seeking their advice on alternative methods.

It is suggested to place concluded projects that have been evaluated as successful by the accompanying expert with a short presentation of its areas of applicability and the contact data of the project leader on the newly established platform swiss3rnetwork.org.

With regards to implementation measures such as validation, commercialization and further propagation, project follow-up must extend the date of publication. This is not how funding is currently handled, but with regards to funding 3R-projects, it is something that must change.

9.5.5 Open access

The successful and widespread implementation of 3R relevant methods is a matter of public interest.

If the accompanying expert of the AfR as well as the project leader agree that further measures for implementation are desirable, doable and promising, the AfR may call on a budget reserved especially for this purpose, e.g. at SNSF (see suggestion below). It remains to be discussed whether only SNSF should have that opportunity or also other funding organizations, such as the Animalfree Research.

9.5.6 Education

Education of students in the matters of the 3R concept is a vital issue. As could be demonstrated, less than half of all participants of the Module 1 course have ever heard of the 3R concept. Knowledge of this concept has to be made obligatory in their education. In addition, it has to be taken care that in the courses, funding bodies are adequately presented.

Political pressure should be exerted in the following areas:

- There should be a call on the SNSF to reserve for instance 0.25% of its annual budget to support implementation of projects that are to be considered successful and promising. The SNSF is a member of the European Science Fund ESF, which has explicitly called on its members to endorse the 3R concept and include it into its funding guidelines. It can therefore not ignore its responsibility in these matters.
- More funding: in 2016 and 2017, and after the closure of the 3RRF, with the AfR being a privately funded body, there was no governmental budget going explicitly to 3R-relevant work at all.
- Endorse open access to publications: with regards to basic research, which is still largely - if not exclusively - funded by tax money, it is actually inconceivable that the

resulting publications are not accessible to the public. In an extreme scenario, a private person that is interested in science first pays for the generation of the data, then again for the licenses that enable universities´ libraries to access the publications (without being in the least capable of affording such a license for private use), and then a third time for actually being allowed to download a paper they are interested in, e.g. from the journal´s website.

- In addition, 3R-relevant papers must be published open access in order to enable the members of the authorities approving animal experimentation to read them and eventually be able to question / contest an applicant´s response that for his or her purpose, no adequate alternative methods are available.
- Visibility: It is suggested to assume a more active role within the scientific community concerned with these issues, a role that the future 3R Competence Centre 3RCC should fulfil. An idea might be a regular workshop together with representatives of other 3R-funding bodies in order to discuss recent developments, problems and possible solutions. Another approach might be the co-organization, or the organization of an entire session, on a current pressing topic, at the European Congresses on Alternatives to Animal Testing or the World Congress on Alternatives and Animals in the Life Sciences. Furthermore, the AfR´s scientific advisor could present concluded projects on a regular basis on these occasions.