Genome Editing using CRISPR/Cas9 as Tool for the Treatment of Breast Cancer

Genomeditierung mit CRISPR/Cas9 als Behandlungsmethode von Brustkrebs

Vorwissenschaftliche Arbeit

verfasst von

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Abstract

CRISPR/Cas9 is a new genetic engineering method that enables scientists to edit the genome in a very simple and targeted fashion. In the last years, genome editing has become fundamental in cancer research and treatment. The aim of this paper is to evaluate the impact of this novel tool, CRIPSR/Cas9, on the range and quality of strategies for fighting against breast cancer. That includes the enhancement of current treatment methods and generally aims at individualizing treatment plans. Targeted therapy can be achieved by sequencing the patient's tumour exome and then specifically modifying the epigenome or the immune system. Furthermore, the option to prevent genetically-inherited breast cancer by intervening in the germline with CRISPR/Cas9 will be discussed. This leads to a general consideration of ethical issues related to CRISPR/Cas9. In order to determine the feasibility of CRISPR/Cas9-mediated gene therapy in Austria, the Austrian law and the attitude of the Austrian population towards this topic will be taken into account.

Prologue

Generally being interested in science, I stumbled across a ScienceBlog post about CRISPR/Cas9. I directly took interest in the topic and later decided to dedicate my pre-scientific paper to it. The choice to investigate its effects on the options to treat breast cancer, was a rather personal one. My grandma defeated breast cancer with the help of radiation and hormone therapy only to now suffer from severe pulmonary fibrosis and not being able to live without additional oxygen supply anymore. I therefore devoutly hope that in the upcoming years breast cancer treatment options will advance - with the help of CRISPR/Cas9- and such severe side effects can be avoided.

I want to thank all the people who supported me during this work. To name only a few of them: my academic advisor, Natascha Rauch, who helped me through the whole process and gave me indispensable guidance and advice. Not to forget that she agreed to let me write the whole paper in English, meaning that she did not only have to deal with the thematic itself but also subject specific vocabulary. Speaking of English, also a great thanks goes to my auntie Julia Wendel for helping me with one or two pieces of vocabulary and grammatical correctness. Lastly, I owe special thanks to Dr. Axel Mündlein from the VIVIT Institute in Dornbirn/Austria who made time for my project not once, but twice. We had a conversation in January 2018 which helped me to get into the topic and he was, later in my writing process, once again there for me to answer my questions about breast cancer and CRISPR/Cas9.

Feldkirch, 20.02.2019 Stephanie Wendel

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1. Introduction

> "Breast cancer is the leading diagnosed cancer for women globally." (Yang, et al., 2018)

Breast cancer affects a lot of people, especially women. In Austria, it is the most common cancer for women, making up around 30% of all those affected by cancer. It is furthermore the leading cause of cancer-related death for women, with 17% total (cf. Statistik Austria, 2018) Still, when Austria is compared to other European states, it shows one of the lowest breast cancer mortality rates. With treatment methods constantly evolving, the life-expectancy for breast-cancer diagnosed patients is getting longer and longer. In Austria 61% are still alive after 5 years. (cf. APA, 2018)

However, current therapies include a lot of harmful side effects that may decrease the quality of life significantly. The combination of Chemotherapy, Radiotherapy and Surgery can be a rather aggressive one, possibly having long-term effects as a result. Therefore, scientists are now trying to develop novel therapy forms. As cancerous cells are mutated cells, often the result of changes to the genome or epigenome, resurrecting the DNA could be a cure for cancer. (cf. Khan, et al., 2016)

CRISPR/Cas9, a genome editing method discovered in 2012 (cf. Jinek, et al., 2012), opens up numerous new opportunities concerning the complexity and also expensiveness of editing DNA.

For this reason, this work will deal with the CRISPR/Cas9 technique by examining its discovery and how it works more closely. Moreover, its main benefits as well as its drawbacks and especially its risks in the therapeutic use will be evaluated.

On this basis the impact of CRISPR/Cas9 on breast cancer treatment and its range of possible methods will be examined. The main aim of this work is to analyse how realistic breast cancer treatment via CRISPR/Cas9 would be. In order to do so, it will be consulting mostly scientific papers from various journals. As the method

and its application to cancer is still in its early stages, I will also evaluate the practicability of hypothetical treatment options. Most of the studies mentioned in this paper, are still ongoing. This means that the upcoming years will show if the different approaches are efficient and ready for broader application.

To get a better insight into breast cancer research I consulted and interviewed Dr. Axel Mündlein from the VIVIT Laboratory in Dornbirn/Austria. VIVIT investigates, among other projects, the impact of inherited genome mutations on breast and colon cancer.

In order to evaluate the practicability of CRISPR/Cas9-based treatments, I will also take ethical questions into account. This includes questions about the right to intervene in not only somatic but also germline cells. Moreover, legal feasibility in Austria will be examined. In the end, I present a survey conducted by the “market Institut” in Linz/Austria to understand the general attitude of the Austrian population towards genome editing.

2. Overview of CRISPR/Cas9

In 1973 biochemist Herbert Wayne Boyer and geneticist Stanley Cohen discovered a way to edit the genome of living organisms. Genome editing has had an enormous effect on the progress in modern medicine ever since. Multiple inventions thanks to genome editing have opened a whole new world of possibilities in modern medicine. However, the possibilities were limited for a long time due to different imprecise methods which were difficult to be produced in large amounts. (cf. Knox, 2017)

These difficulties were not solved until the discovery of "CRISPR/Cas9". This technique was originally discovered as part of the immune system of various bacteria and archaea[1]. It is often described as "genome scissors" since one part of the CRISPR/Cas9-complex, more specifically the enzyme "CRISPR-associated9" (for short Cas9), is able to cut DNA. This enzyme is told by the so-called "guide-RNA[2]" where it has to cut in order to achieve the wanted effect. Being able to guide the executing enzyme scientists can now more precisely insert, delete, modify or repair genes. (cf. Taxler, 2017)

The other part, referred to as "CRISPR" (abbreviation for "clustered, regularly interspaced, short, palindromic repeats"), represents the memory of bacterial and archaeal immune systems. The information about already defeated viruses is archived in "spacers[4]" and can be resorted to in case of further infection. The intruder can then more quickly be detected and destroyed. As the information is inherited by the next generation the progeny is also protected against the virus. (cf. Doudna, 2015)

[1] archaea = *also:* archaebacteria; prokaryotic organisms
[2] RNA = nucleic acid[3]; encodes genetic information; similar to DNA, but single-stranded
[3] nucleic acid = hypernym for DNA and RNA; built of "nucleotides" (= phosphate residue + [deoxy]ribose + base[9])
[4] spacer sequence = piece of viral DNA that is embedded in the bacterial genome

2.1. Discovery of CRISPR/Cas9

Back in 1978, when a Japanese team of scientists at Osaka University discovered what is now called a CRISPR locus during their research on *Escherichia coli,* it was unfamiliar to them. (cf. Ishino, et al., 1987) Francisco Mojica found a similar structure whilst examining an archaeal[1] microbe in 1993. He took interest in studying it more closely and was later on the first to define the hallmarks of a CRISPR array and establish the term "CRISPR". (cf. Mojica, et al., 1993)

When Mojica found the connection between the DNA structure mentioned in the Japanese paper and his own discovery, he speculated on its function. Since similar patterns of DNA were found in bacteria as well as in archaeal microbes, two non-related species, he concluded that CRISPR had to hold a crucial function in prokaryotes[5]. (cf. Mojica, et al., 1995) His assumption that CRISPR was part of an adaptive immune system against viruses was proven right by Phillipe Horvath in 2007. Their experimental demonstration of adaptive immunity also led Horvath and his colleagues to the conclusion that Cas9 was likely the only protein needed to interfere. (cf. Horvath, et al., 2007)

Cas9 itself was discovered in 2005. Alexander Bolotin found an array that lacked some of the genes known until then. Instead it was provided with an unprecedented gene, which encodes the protein Cas9. Moreover, during his studies he noted a recurring sequence at the end of each spacer[4]. This "protospacer adjacent motif" (PAM for short) is important for the functioning of the CPISPR/Cas9 system as it is required to distinguish the spacer from the actual viral DNA sequence the organism wants to eliminate. (cf. Bolotin, et al., 2005)

In 2008, John van der Oost and his colleagues found out about the enzyme Cas9 being led by small RNAs[2], transcribed from the spacer sequences. (cf. Brouns, et al., 2008)

[5] prokaryotes = organisms whose cells do not have a nucleus; *opposite:* eukaryotes

Also, a team of scientists at Northwestern University in Illinois understood that the CRISPR/Cas9 system targets DNA, which evidently is a pivotal point when wanting to harness it for genome engineering in eukaryotes[6]. (cf. Marraffini & Sontheimer, 2008)

Referring to the findings of Garneau et al. two years later, the Cas9 enzyme induces double-stranded breaks[7] in the DNA at precise positions, more specifically 3 nucleotides away from the PAM-sequence. Hence, it also uses PAM as a guidance. (cf. Garneau, et al., 2010)

When Emmanuelle Charpentier partnered up with Jennifer Doudna in 2011, they found the missing piece to fully understand the natural CRISPR/Cas9 mechanism by discovering the tracrRNA, another element of the CRISPR/Cas9 complex. (cf. Deltcheva, et al., 2011) Furthermore, at about the same time, Virginijus Siksnys and colleagues from Lithuania noted that CRISPR/Cas9 can also function in other species. (cf. Sapranauskas, et al., 2011)

Back then, the team of scientists around Doudna and Charpentier had no intention to find a device able to control genetic material. Their starting research examining the effective bacterial defence system against viruses was mostly reasoned in pure curiosity, explains Jennifer Doudna. (cf. Doudna & Sternberg, 2017) However, their research led them further and in 2012 they published a paper showing how to utilize the CRISPR immune system as a genetic engineering tool. (cf. Jinek, et al., 2012)

In 2013, the team around Fheng Zhang from Broad Institute in Cambridge, Massachusetts submitted a patent application concerning genome editing with CRISPR/Cas9 in eukaryotic cells. (cf. Cong, et al., 2013) At the same time George Church's lab at Harvard University had similar findings. (cf. Mali, et al., 2013)

[6] eukaryotes = organisms whose cells do have a nucleus; *opposite:* prokaryotes
[7] double-stranded break = *also:* DSB (short form); both DNA strands get cut simultaneously

A patent fight between those three parties broke out, ending with most of the intellectual property being adjudicated to the Broad Institute. (cf. Cohen, 2017)

2.2. Functional principle of CRISPR/Cas9

The principle to edit DNA with CRISPR/Cas9 is based on how the bacterial or archaeal[1] adaptive immune system is executed when the organism is attacked by a phage[8]. (cf. Taxler, 2017)

In the bacterial/archaeal DNA there is a certain structure to find, containing "multiple copies of a near-perfect, roughly palindromic, repeated sequence of 30 bases[9]" (Lander, 2016), called CRISPR. In between the recurring sequences there are pieces of viral DNA, referred to as "spacer DNA[4]". Nearby, the Cas-genes encode proteins, including Cas9. (cf. Max-Planck-Gesellschaft, 2018)

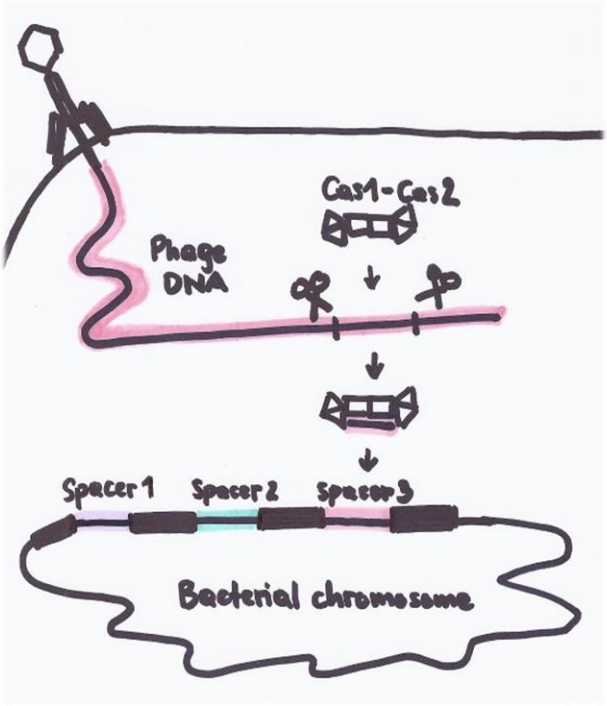


Figure 2: Acquisition of phage sequences (own graph)

When a phage injects viral DNA in the cell of the host, the CRISPR array acquires pieces of the viral DNA by cutting it out with help of the proteins Cas1 and Cas2. The result is a so-called protospacer that is then embedded in the CRISPR pattern. (cf. Lo, 2017) [see Figure 2]

In the CRISPR pattern, CRISPRs and spacers are alternating. It can thus be divided in smaller sections that, when transcribed, are termed crRNA (coming from CRISPR-RNA) and are compounds of a CRISPR sequence and a piece of viral DNA.

[8] phage = *also:* bacteriophage; virus that infects bacteria and archaea to propagate in its host
[9] bases = *more specifically:* nucleic acid bases; components of DNA and RNA; always bind to their complementary base (Adenine – Thymine/Uracil; Cytosine – Guanin)

When bounding to Cas9 the crRNA is followed by the tracrRNA ("trans-activating-crRNA") which leads the executing enzyme and the crRNA to their target location. In conclusion, the CRISPR/Cas9 complex consists of the crRNA, the tracrRNA and the enzyme Cas9. [see Figure 3] (cf. Max-Planck-Gesellschaft, 2018)

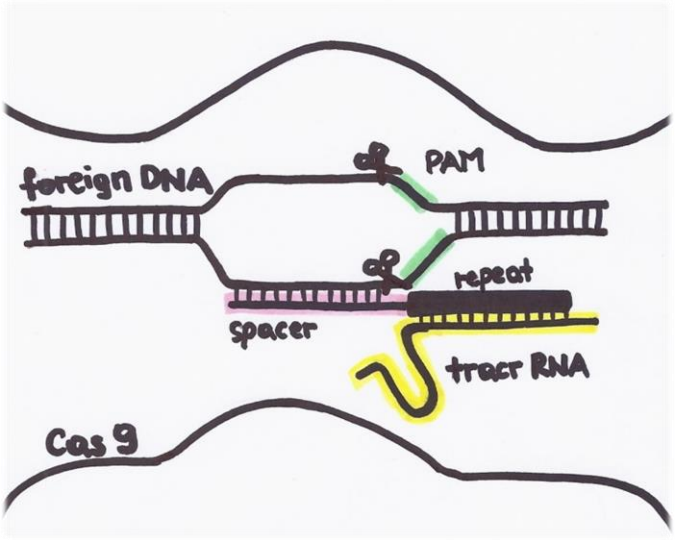


Figure 3: Native CRISPR/Cas9 complex (own graph)

This CRISPR/Cas9 complex is controlling the prokaryotic cell and searches for intruders by comparing and looking for sequences matching the spacer RNA. In order to distinguish the viral DNA from the crRNA the complex specifically tries to detect a 2 to 6 base pairs long sequence, located next to the protospacer sequence in the viral genome. This "protospacer adjacent motif" (PAM) firstly accelerates the process as it is followed by the viral protospacer sequence and secondly keeps the archaea/bacteria from destroying its own genetic material. The PAM is also indispensable for Cas9 to be able to bind with viral DNA and in further consequence, cut it. (cf. Lo, 2017)

When the match is found the viral DNA is unwound sectionally. Then RNA^2-DNA hybridisation[10] is initiated as the spacer RNA bounds with the viral DNA due to hydrogen bridges. This introduces a double-stranded break[7], which is often visualised as "cutting the DNA". (cf. Doudna, 2017)

[10] hybridisation = process when two complementary nucleic acid strands congregate to a double strand

Normally, in bacteria double-stranded breaks lead to the degradation of DNA. In this way CRISPR/Cas9 destroys the viral DNA and thus successfully eliminates the risk to get infected by this virus. This is why, in bacteria and archaea, CRISPR/Cas9 is such an efficient adaptive immune system. [see Figure 4]

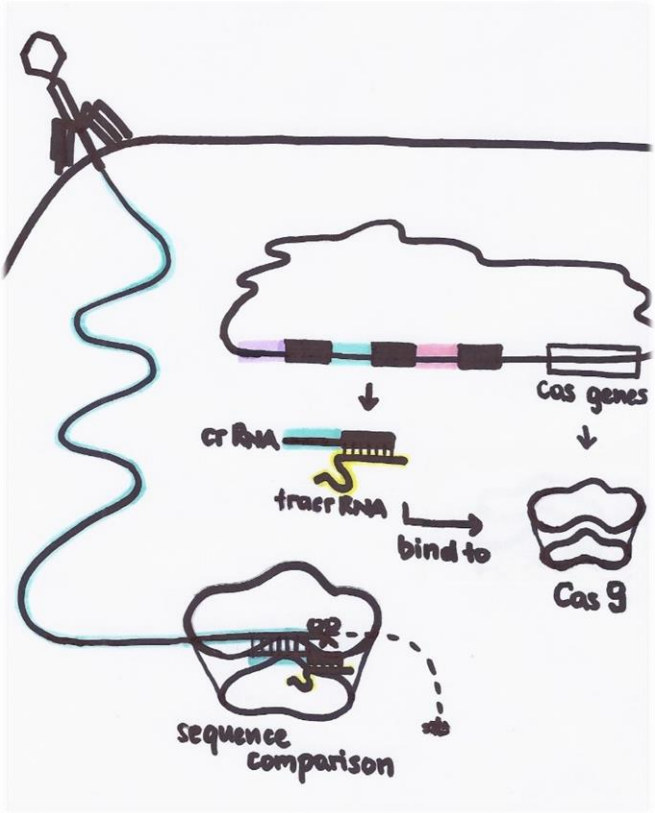


Figure 4: CRISPR/Cas9 as adaptive immune system (own graph)

Eukaryotic cells, however, developed a machinery to repair DNA damage. To do so, they either integrate a whole new piece of DNA or fix it with a small change. For this reason, introducing double-stranded breaks is generally a good way to edit the genome. Once the DNA is broken, there are several options: inserting a new piece of DNA into the target, modifying and therefore repairing the genome or e.g. completely deleting genes. (cf. Doudna, 2017)

The method to cause double-stranded breaks was used for genetic engineering even before the discovery of the CRISPR/Cas9 method. With CRISPR/Cas9, however, it is now possible to initiate them more precisely and easily. In addition, the construction of the complex is relatively simple. When CRISPR/Cas9 is used for genome editing, there is a slight difference in regard to the structure. [see Figure 5]

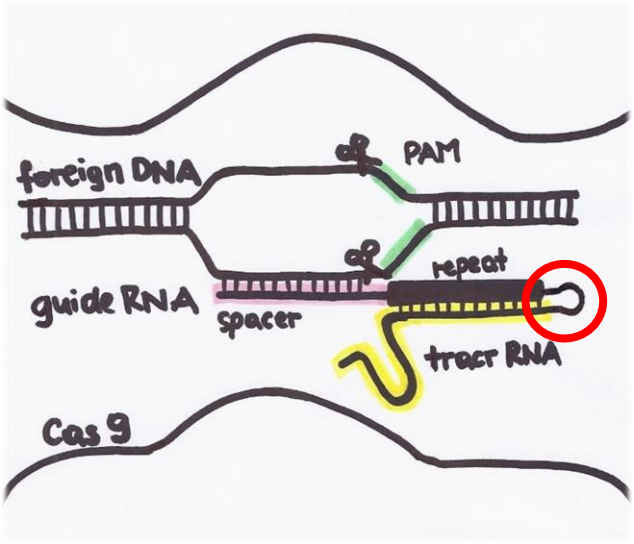


Figure 5: CRISPR/Cas9 complex when used for genome editing (own graph)

The native immune system works on basis of viral DNA whereas, for genetic engineering, as it is aiming at a specific target instead of a virus, an artificial guide RNA (=gRNA) is used. Therefore, also the PAM varies from target to target, depending on what lies next to what one wants to cut. (cf. Synthego, 2018)

2.3. Positive and negative aspects of CRISPR/Cas9

In 2012, 126 scientific papers were published on CRISPR/Cas9. Within 5 years the number rose up to 2155 in 2017 and continues to grow. Evidently, this new genome editing method provides scientists with a lot of benefits such as relatively simple construction, low costs, high versatility and high functional efficiency. (cf. Cohen, 2017)

Contrarily to its predecessors TALENs[11] and ZFNs[12], in order to produce a CRISPR/Cas9-complex no protein engineering steps are needed. This facilitates the process of producing and testing several gRNAs in order to find the fitting gRNA for the target. Nearly every gRNA can be produced straightforward, since the whole genome sequence of most domestic organisms is known.

The circumstance that no extraordinary measures are required for the synthesis of a CRISPR/Cas9-complex makes this technique so inexpensive. Therefore, financially, it comes within the reach of nearly every molecular laboratory. This makes it possible to gather large libraries of gRNAs to which one can resort later on. In addition, basic research is supported.

Another reason why the amount of publications has augmented that rapidly in the past years, could be that information about the technique is accessible. Newcomers are encouraged and supported by for example active discussion groups. (cf. Bortesi & Fischer, 2015)

[11] TALEN = “transcription activator-like effector nuclease”, artificial protein used for genome editing
[12] ZFN = “zinc finger nuclease”, artificial protein used for genome editing

A unique attribute of CRISPR/Cas9 is that until now it is the only discovered genome editing method that can target methylations[13] on the DNA, which enables it to also edit the epigenome[14]. This gives the scientist the ability to switch certain genes on or off. (cf. Moses, et al., 2018)

Since the Cas9-enzyme can be combined with basically any number of gRNAs, multiple targeting at the same time is now possible. In this way, double-stranded breaks[8] can be induced at several sites in the genome, which can be of use for example when dealing with parallelly working signalling pathways.

The main drawback of the CRISPR/Cas9 method are the so-called “off-target effects". This term describes the cleavage of a DNA-sequence, that was not intended to be targeted. This can happen if the off-target sequence is similar to the gRNA. Scientists see the reason for this process in the original purpose of CRISPR/Cas9. A natural lack of specificity lets the bacterial immune system take eventual point mutations[15] in the genome of the invading virus into account. But what has proven itself useful for bacteria is rather problematic when it comes to harnessing CRISPR/Cas9 for genome editing. Once exchanged, added or deleted a base can make all the difference and often decide between a healthy or a disease-determined life.

In early research off-target effects occurred relatively frequently. Therefore, researchers have come up with several different solution approaches. Firstly, there are platforms helping scientists to choose the right gRNA. By calculating the probable outcomes, taking off-target effects into account, the specificity can be predicted, and the most suitable gRNA selected. Another approach is to induce DSBs that have a base[9] overhang. This enhances the specificity, because there are more bases to be recognized for the base pairing. (cf. Bortesi & Fischer, 2015)

[13] methylation = annealing of one or several methyl group(s); methylation of the DNA is responsible for cell development and cellular differentiation
[14] epigenome = genetic information that cannot be ascribed to the actual DNA; epigenetic changes = heritable changes in gene expression due to environmental impacts; (in)activation of certain genes through e.g. methylation
[15] point mutation = one base in the genetic code is exchanged, added or deleted

Pawluk et al. found another way to improve specificity. Their method is related to the original purpose of CRISPR/Cas9 as it harnesses so-called “anti-CRISPRs”. In nature, bacteriophages[8] use anti-CRISPS to fight the bacterial immune response and they can therefore be used as “off-switches” for CRISPR/Cas9-mediated genome editing. (cf. Pawluk, et al., 2016)

Apart from the off-target effects, there are more barriers in therapeutic use. Until now, the frequency of edited sites in several cells is still low, which makes it difficult to apply *in vivo* therapy. Moreover, the delivery into the right site in the body is challenging, since the enzyme Cas9 is quite big.

This is one reason why *ex vivo* therapy methods are often preferred. The delivery is not that challenging and in addition, the edited cells can be checked directly and, in case that any off-target effects were detected, also be corrected. (cf. Moses, et al., 2018)

3. Possible Effects of CRISPR/Cas9 on the treatment of breast cancer

> "Cancer is caused by a series of alterations in genome and epigenome[14] mostly resulting in activation of oncogenes or inactivation of cancer suppressor genes."
> (Khan, et al., 2016)

As cancer is genetically-induced, genome editing could have momentous effects on future therapies. Up until now, different treatment methods, such as chemotherapy and radiation have improved and became more efficient, but the side effects remain harmful and can have long-term effects on the quality of life. Using CRISPR/Cas9 to correct the cancer-related mutations would have the potential to be a reliable long-term solution specifically targeting the cause of the disease. (cf. Khan, et al., 2016)

Doudna states that with CRISPR/Cas9 every genetic disease for which the responsible mutation is known has the potential to be treated more individually. Furthermore, using CRISPR/Cas9 technology is not only about treating diseases but also preventing them. Congenital diseases affect large parties of our population and genome editing could stop the process of inheriting them further on. However, to do so, scientists would have to modify the human germline[16]. As a change in the germline is heritable, an altering would affect all further generations. (cf. Doudna & Sternberg, 2017)

3.1. Treatment methods of breast cancer

The following treatment strategies have constantly been evolving in the past years, enabling a high survival chance for breast cancer patients. However, due to aggressive approaches severe side effects can occur not only during treatment but may affect patients for the rest of their lives.

[16] germline = evolution of a fertilized egg cell to germ cells (=sexual cells)

Usually, several treatment methods are used in combination. There is a difference between systematic treatments, which affect all the cells in the body, and more targeted therapies, that are either applied locally or only aiming at cancer cells. The elected methods evidently depend on the type of breast cancer the patient is suffering from and whether it has formed metastases.

There are three main categories of breast cancer. The most common type, affecting around 2 out of 3 patients, is to be hormone receptor-positive. One can either be ER- (oestrogen receptor) or PR- (progesterone receptor) positive or be both. The so-called HER2[17]-positive breast cancer is contested in approximately around 1 out of 5 patients. In this case, the cancer cells express a surplus of HER2 proteins. Oncologists speak of "triple-negative breast cancer" if neither ER receptors, PR receptors nor excess of HER2 occurs. This is the case in about 15-20% of breast cancer incidents. (cf. Kingston & Johnston, 2016)

3.1.1. Surgery

When talking about surgery as treatment method for breast cancer one needs to distinguish between a "breast conserving surgery", a total breast removal or the procedure of extracting surrounding tissue, for instance the lymph nodes.

A so-called lumpectomy tries to conserve as much of the natural breast tissue as possible, removing only the cancerous part of the breast. Evidently, the feasibility of this type of surgery depends amongst other factors on the size and location of the tumour.

When the tumour has grown too big, or for other individual reasons, a mastectomy is elected for the patient's treatment plan. During this surgery the entire breast is removed, sometimes including the surrounding tissue. In order to eliminate the risk of developing cancer in the second breast too one could decide to have a double mastectomy, meaning to remove both breasts. A mastectomy is often followed by breast reconstruction. (cf. Cancer Research UK, 2017)

[17] HER2 = "human epidermal growth factor receptor 2"; oncogene that stimulates cell proliferation

In order to figure out to which stage the cancer has advanced, one or several lymph nodes can be removed and later examined in the laboratory. In case the cancer has spread, most or all nodes in the axillary region will be removed.

Often surgery is accompanied by radiotherapy. It is most likely to be treated with radiation before or after having breast conserving surgery. (cf. American Cancer Society, 2016)

3.1.2. Radiotherapy

During Radiotherapy high-energy rays, usually x-rays, are used to fight the tumour. This treatment strategy targets cancer locally, meaning that the body is only rayed in the cancerous area. One can differentiate between external and internal radiation, which may also be called brachytherapy.

With external radiation the radioactive source is a machine outside the body that focuses the beam radiation on the tissue affected by cancer. Often external radiation is conducted after surgery in order to lower the reoccurrence risk.

During internal radiation the source of radiation is placed directly into the breast. Throughout a thin hollow tube or an inflatable balloon a small radioactive pellet can be brought into the breast tissue, where it releases doses of radiation. This procedure, along with external beam radiation, is often recommended in the surgery site after a lumpectomy. The option to limit radiotherapy to internal radiation is often not promising due to the tumour's size and its location. (cf. American Cancer Society, 2017)

Radiation does not only affect cancer cells but all the cells in the breast area. Therefore, long-termly seen, the patient might develop chronic radiation pneumonitis[18] which leads to permanent chough and breathlessness and can result in

[18] chronic radiation pneumonitis = disease condition caused by changes to the lung tissue resulting in constant shortness of breath

pulmonary fibrosis[19]. After radiation one is also in greater danger of developing osteoporosis[20]. (cf. Cancer Research UK, 2017)

3.1.3. Chemotherapy

During chemotherapy anti-cancer drugs are either injected in one's bloodstream and in this way circulate throughout the body or taken as tablets. Usually chemotherapy drugs are given in combination with other treatment methods, primarily surgery.

Having chemotherapy before surgery is called "neo adjuvant therapy". This might be suggested when having a locally advanced tumour. The aim then is to shrink the mass in order to have a less extensive surgery, so for example a lumpectomy instead of a complete mastectomy, and to lower the reoccurrence risk.

When it is feared that the cancer could have disseminated to different parts of the body, an "adjuvant therapy", so chemotherapy after surgery, is given. Also, its intention is to prevent the cancer from coming back. In case of advanced cancer, adjuvant therapy can help control the tumour and improve the patient's quality of life, however, curing it is more unrealistic.

Side effects of this therapy might be, among others, weakening of the immune system. Chemotherapy can furthermore lead to changes in menstrual periods, temporarily or long-term, which can result in infertility if the oestrogen production is stopped. (cf. Cancer Research UK, 2017) Generally, there is a higher risk to develop osteoporosis[20] and suffer from bone loss, heart- and nerve damage. (cf. American Cancer Society, 2017)

[19] pulmonary fibrosis = disease condition caused by irreversible scarring of the lung tissue; often result of chronic radiation pneumonitis

[20] osteoporosis = weakening of the bones, which e.g. increases the risk of having bone fractures

3.1.4. Hormone therapy

This therapy strategy requires that the cancer is ER- or PR- positive, which means that the cancer cells have oestrogen or progesterone receptors to which the hormone can attach. If this is the case, high female hormone levels support the proliferation of cancerous cells. (cf. American Cancer Society, 2017) Treatment using hormones thus aims at lowering the oestrogen or progesterone levels and is therefore classified as systematic therapy.

Hormone therapy can be used before or after surgery, to slow the growth or decrease the risk of the cancer coming back. It is usually ingested as tablet for at least 5, often up to 10 years. (cf. Cancer Research UK, 2017)

There are several different treatment approaches utilizing hormones. SERM drugs, these are “selective oestrogen receptor modulators”, block the oestrogen receptors of the cancer cells. This type of drug may increase the likelihood of developing uterine cancer and affects the patient’s bones.

SERD drugs or “selective oestrogen receptor degrader” are more aggressive, as they do not only block but also damage the hormone receptors. They are used if other more common hormone drugs failed. Working throughout the whole body, they are only approved for patients who already had their menopause and can have osteoporosis[20] as a side-effect.

Aromatase inhibitors aim at suppressing the oestrogen production. The enzyme aromatase continues to produce oestrogen after the menopause. It is therefore mainly used for post-menopausal patients or in combination with ovarian suppression for those who did not yet have their menopause. Ovarian suppression can either be reached by surgery, so removing the ovaries, as a side-effect of chemotherapy drugs or by utilizing hormones, which stop the ovaries from producing oestrogen and cause a temporary menopause. (cf. American Cancer Society, 2017)

3.1.5. Targeted cancer drugs

Targeted cancer drugs seek to specifically target cancer cells and might be utilized along or instead of chemo- and hormone therapy. As the drugs aim at one particular cancer cell type, different methods have been developed to treat the diverse types of breast cancer. (cf. American Cancer Society, 2018)

In order to weaken HER2[17]-positve breast cancer monoclonal[21] antibodies can be harnessed. In general, antibodies aim at finding particular proteins on cells. The idea behind this strategy is that they attach to the HER2 proteins to stop the cancer cell from growing and dividing. (cf. Cancer Research UK, 2017) Furthermore, HER2 proteins can be targeted by kinase inhibitors. Since any protein activation or inactivation is under the control of certain kinases (cf. Gross, et al., 2015), the surplus of HER2 could thereby be stemmed. This type of cancer drugs can lead to heart damage.

Proliferation of hormone receptor-positive breast cancer can be slowed down by drugs blocking cyclin[22]-dependant kinases (=CDKs). These proteins play an important role in the cell cycle and are thought to be responsible for cancer cells eventually becoming resistant to hormone therapy. (cf. Kingston & Johnston, 2016) Moreover, dysregulation of the cell cycle is a typical reason for cancer spread and further metastases development. Since CDK inhibition allows to recontrol the cell cycle, there are now studies to use it against metastatic hormone receptor-positive breast cancer. (cf. Mayer, 2015)

To enhance the treatment of cancer caused by BRCA ("BReast CAncer gene") mutations [for further explanation see 3.4] drugs functioning as PARP ("poly ADP-ribose polymerase") inhibitors can be harnessed. PARP proteins are involved in various cell processes, such as DNA repair and apoptosis[23]. Suppressing them therefore

[21] monoclonal = *here:* only one type of antibodies is used
[22] cyclin = protein that has impact on the regulation of the cell cycle
[23] apoptosis = controlled, self-induced cell death

weakens the, due to the dysfunctional BRCA, already feeble DNA repair mechanism, which often results in death of the tumour cells. (cf. Kingston & Johnston, 2016)

3.1.6. Immunotherapy

There are different approaches, making use of different parts of the immune system. One of them is T-cell[24] transfer, which will later be mentioned in 3.3.3.

Kingston & Johnston from the Royal Marsden Hospital in London propose a new immunotherapy method to fight triple negative breast cancer. TNBC cells sometimes manage to undergo the immune system by expressing the protein PDL-1. As soon as PDL-1 binds to certain T-cell receptors, the immune response is inhibited. To deal with this problem, the PD-1 receptors on tumour cells can be blocked. Furthermore, PDL-1-suppressor medicaments have been developed. (cf. Kingston & Johnston, 2016)

3.2. Requirements for the use of CRISPR/Cas9

According to the findings of Bolotin et al. in 2005, for CRISPR/Cas9-based immunity, and in further consequence genome editing, it is inevitable that the DNA sequence of the spacer[4] matches the ones of the targets precisely. (Bolotin, et al., 2005) Thus, in order to correct a mutation or destroy a gene, one has to know exactly the DNA sequence of the aim. Only then is it possible to build a functioning guide-RNA that shows Cas9 the right way. In order to erase a breast cancer-related mutation with CRISPR/Cas9 it would therefore be necessary to know which mutations caused the cancer. Mündlein from the VIVIT laboratory in Dornbirn/Austria explains that, thanks to large sequencing projects, scientists nowadays are familiar with the complete human genome sequence. Therefore, and because of the circumstance that gRNA is short, he sees no problem in targeting any breast cancer associated gene. (cf. Mündlein, 2019)

[24] T-cell = cells of the immune response; responsible for adaptive immunity

3.3. Different approaches in treatment with CRISPR/Cas9

Since there are different breast cancer types, all induced for another reason, the methods harnessing CRISPR/Cas9 are more or less efficient for the different cancer subtypes. However, in general, cancer progression is usually addicted to several factors. Through CRISPR/Cas9 one has influence on the most common cancer drivers like transcription aberrations, splicing mistakes, faulty translation, protein degradation or misfolding, uncontrolled cell proliferation and inhibited cell death.

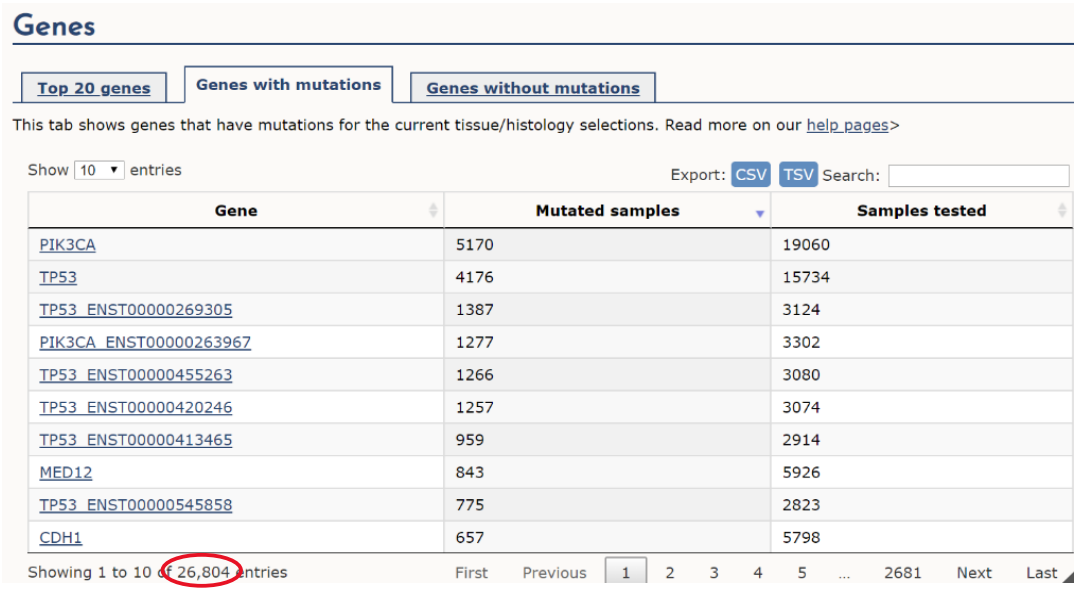
In addition, CRISPR/Cas9 can help to improve already existing methods or develop treatment methods for breast cancer that are based on strategies for other types of cancer. Moreover, investigating cancer in order to develop novel treatments options can be facilitated by for example modelling cancer in mice through CRISPR/Cas9 induced alterations in its genome. (cf. Moses, et al., 2018)

3.3.1. Erase mutations

As cancer is often induced due to either genetically-inherited or environmentally-related mutations, erasing all those mutations could stem the tumour progression.

Up until now several mutations are considered to be removeable using this method. For example, the genes encoding HER2[17] proteins and BRCA (so BRCA1 and BRCA2 mutations) are possible CRISPR/Cas9 targets, opening up the opportunity to preventively correct inherited BRCA mutations [for further explanation see 3.4]. (cf. Khan, et al., 2016)

However, this is only a fractional part of possible mutations related to breast cancer. The difficulty is that there are thousands of genes that could be mutated, like data of the COSMIC database (“catalogue of somatic mutations in cancer”) shows indisputably [see Figure 6]. (cf. Sanger Institute, neg.)



Genes

Top 20 genes | Genes with mutations | Genes without mutations

This tab shows genes that have mutations for the current tissue/histology selections. Read more on our help pages>

Show 10 entries Export: CSV TSV Search:

Gene	Mutated samples	Samples tested
PIK3CA	5170	19060
TP53	4176	15734
TP53_ENST00000269305	1387	3124
PIK3CA_ENST00000263967	1277	3302
TP53_ENST00000455263	1266	3080
TP53_ENST00000420246	1257	3074
TP53_ENST00000413465	959	2914
MED12	843	5926
TP53_ENST00000545858	775	2823
CDH1	657	5798

Showing 1 to 10 of 26,804 entries First Previous 1 2 3 4 5 ... 2681 Next Last

Figure 6: Breast cancer gene mutations, showing 1 to 10 of 26 804 entires (Sanger Institute, neg.)

Nevertheless, due to next generation sequencing technologies, it would be possible to sequence the whole tumour relatively inexpensively, meaning that, theoretically, there are no restrictions in mutation detection. However, there are a few peculiarities of cancer that should be taken into account, states Mündlein. Firstly, as there are not only driver mutations but also passenger mutations, the impact of a mutation on cancer cell proliferation, and thus the necessity of erasing it, can sometimes be unclear. Furthermore, the progression of cancer is not only supported by gene mutations. Epigenetic modifications and changes in the number of gene copies also play a significant role. (cf. Mündlein, 2019)

> "It should be further noted that, […] tumours can be polyclonal; that means that a tumour may be composed of cells from at least two distinct progenitors with different mutational profiles." (Mündlein, 2019)

This expands the variety of mutations even more and further complicates the identifying process. In addition, correcting the mutation in one copy of the oncogene, is not efficient, as the frequency of CRISPR/Cas9 edited sites in the genome is often low. [also see 2.3.]

3.3.2. Altering epigenome[14]

> "Epigenetic changes defines [sic!] the environment for cancer development." (Khan, et al., 2016)

Those epigenetic modifications highly affect gene expression and thus also cell activity, such as proliferation and differentiation. Furthermore, a lot of cancers are either caused when general methylation[13] no longer functions correctly or by hypermethylation of certain arrays. Khan et al. therefore state that findings to change the epigenome will be a key factor for limiting cancer in the future. (cf. Khan, et al., 2016)

To edit the epigenome, a "dead" Cas9 protein can be harnessed. This "dCas9" has, just as Cas9, the ability to bind to DNA, however, it does not induce DSBs[8]. It can therefore be used to alternate epigenetic marks without affecting the patients' actual DNA.

Moses et al. suggest the idea of harnessing epigenetic engineering via CRISPR/Cas9 in order to activate tumour suppressor genes and on the other hand silence oncogenes. As tumour suppressor genes are often hypermethylated and are therefore not working, reactivating them should bring the wanted effect of cancer suppression. Based on this approach, methods using ZFNs[12] or TALENs[11] have already been tested. The main problem of these methods lay in the lack of specificity, which can be solved by using dCas9. Moses et al. mention especially BRCA mutations as a suitable target. (cf. Moses, et al., 2018)

Another approach involving epigenetics is presented by Khan et al. Their starting point lies in the discovery of non-coding RNAs[25], which are associated with the epigenetic regulation of cell growth. (cf. Khan, et al., 2016) Hsu et al. recently did research aiming at this direction, which showed promising results. They successfully

[25] non-coding RNA = does not encode proteins, but can control gene expression

targeted non-coding RNAs with CRISPR/Cas9. In this way, they were able to control cell proliferation and should be able to stem it in the case of cancer cells. (cf. Hsu, et al., 2014)

Furthermore, CRISPR/Cas9 is considered a solid device for high specificity epigenetic alterations. This could enable the approval for epigenetic drugs, that are very efficient against cancer but were previously declined due to extensive side effects. (cf. Moses, et al., 2018)

3.3.3. Improved Immunotherapy

The aim of immunotherapy is to fight the cancer hallmark of immune destruction. Often, genetic material that enhances the immune response against cancer cells is inserted in immune cells. In general, there are two options. Either engineered T-cells[24] of the patient itself are used or a donor provides suitable cells, for example if the patient's immune system is too weak. In this case, a so-called "graft-versus-host disease" can occur. This means the donor cells recognize their new environment as foreign and attack the body instead of the cancer. By editing the donor cells and removing a specific molecule, one can make them believe to be endogenous. All these procedures could now be conducted more easily with CRISPR/Cas9. (cf. Walsh, 2017)

Another approach would be to cut out the genes that encode cancer supporting elements. One example would be the gene that encodes the receptor PD-1, which, referring to the findings of Kingston & Johnston, can play a role in triple negative breast cancer proliferation [see 3.1.6]. (cf. Kingston & Johnston, 2016) There is an ongoing phase I trial, that investigates the safety of a lung cancer therapy harnessing PD-1 deficient T-lymphocytes engineered with CRISPR/Cas9. Mündlein states that, if the study will show success in the next phase and augment survival probability, the treatment strategy may be applied to other cancer patients. However, for him, the role of immunotherapy in triple negative breast cancer is not yet clear. (cf. Mündlein, 2019)

Recently, a patient with metastatic breast cancer, was successfully treated with a new type of breast cancer immunotherapy. This new strategy analyses the DNA of the tumour to find mutations that are specific for the cancer cells. The T-cells that recognise these cancer mutations are taken out, cultivated in the laboratory and later reinserted into the host, enhancing the immune response against cancer. The method is still in its beginnings and trials are ongoing. (cf. National Cancer Institute, 2018) At the moment it is not harnessing CRISPR/Cas9, however, screening for mutations can, according to Moses et al., be simplified by using CRISPR/Cas9. In this way, the costs, that are at the moment very high due to the complexity, maybe could be reduced.

Using CRISPR/Cas9 to enhance immunotherapy has an immense benefit in comparison to other strategies harnessing CRISPR/Cas9. As it is an *ex vivo* therapy the edited cells can be examined in the laboratory. Thus, the risk of off-target effects can be reduced to a minimum. Cells that show any abnormality can be sorted out. (cf. Moses, et al., 2018)

3.3.4. Fight resistance against other cancer drugs

Often, patients develop resistance against the cancer drugs they are given. As the resistance is mostly reasoned in anew changes of the genome, like e.g. reversion mutations, CRISPR/Cas9 carries the possibility to encounter the cause of resistance and further re-establish the inhibitory effect against cancer progression.

An example for this is the PARPi – treatment against BRCA-related breast cancer. [for further explanation see 3.1.5] Although patients tend to show improvement at first, some eventually become immune to the PARPi-drugs and the tumour continues to grow. It seems that cancer cells evolve therapeutic resistance due to the removal of the mutation on BRCA1. The BRCA1 protein then remains active in tumour cells, which is why drugs like PARPi, which affect the normally feeble repair mechanisms, are no longer efficient. By re-introducing the mutations with CRISPR/Cas9, defective DSB[7] repair can be re-installed in cancer cells, making them sensitive to PARPi treatment again. (cf. Yang, et al., 2018)

3.3.5. Aid to develop new treatment methods

The first step of developing novel therapy methods is to gain knowledge of the target, which includes genome mapping in order to find the sequences that could be responsible for the cancer.

With CRISPR/Cas9 generating complexes is relatively straightforward. As a consequence, it is also easy to create gRNA libraries, which further facilitates the process of genomic screening. In the cancer research field this makes it easier for scientists to identify oncogenic drivers or sequences that help the cancer to e.g. establish drug resistance. It also enables them to understand processes like metastasis and to find the responsible drivers. Logically, this also simplifies the screening of the patient's tumour exome to further elect an individualized treatment plan.

When wanting to better understand cancer proliferation, often research on the mutations considered to support cancer is conducted. In order to validate one's hypothesis *in vivo*, so the see if they really are cancer-associated mutations, usually mouse models are generated. With CRISPR/Cas9 it is a lot more facile to do so. Firstly, the editing process is accelerated and secondly, it becomes inexpensive. In addition, researchers can create more complex and multi-layered genetic changes. (cf. Moses, et al., 2018) In recent studies, CRISPR/Cas9 was already deployed to model cancer mutations by switching off tumour suppressor genes. (cf. Xue, et al., 2014) As it is easy to simulate various disease patterns, scientists can investigate different hallmarks of cancer via CRISPR modelling. Research has already, amongst others, been done on cancer signalling pathways, coherence between mutations and genome instability and the process of cancer cells becoming resistant to cell death. This provides scientists with excellent means to gain knowledge about tumour evolution, which in further consequence enables them to find better treatment options against it. (cf. Moses, et al., 2018)

To sum up, Moses et al. attach great importance to CRISPR/Cas9 in current and future cancer research:

> "These examples illustrate how CRISPR provides a versatile and efficient gene editing system to study cancer genesis and development [...] and is now established as an indispensable tool in the field." (Moses, et al., 2018)

3.4. Prevention of genetically-inherited breast cancer

There are two different types of breast cancer occurrence: sporadic and spontaneous or familial. Whereas a patient with sporadic breast cancer has no relatives suffering from the same illness, the other patient has a long family history of breast cancer and the disease appears to be inherited. (cf. Mukherjee, 2016) The analysis of a research conducted by Mary-Claire King indicated that a gene is responsible for most of the familial cases. (cf. Hall, et al., 1990) Women carrying a mutated version of this gene, called BRCA1, have a risk of 72% of developing breast cancer until age 80. (cf. Kuchenbaecker, et al., 2017)

In 1995, a second cancer susceptibility gene, BRCA2, was discovered at the Institute of Cancer Research, UK, by a team of scientists around Michael Stratton. (cf. Wooster, et al., 1995) The cumulative breast cancer risk for BRCA2 is 69%. (cf. Kuchenbaecker, et al., 2017) Naturally, the probability rises, if one does not only have a mutation on a single gene but on BRCA1 and BRCA2. (cf. Antoniou, et al., 2003)

The reason for cancer development in both cases is that without correct functioning of BRCA1/2 damage repair of DNA is in distress. (cf. Easton, et al., 2015) Since BRCA1/2 encodes a protein that is crucial to repair a broken DNA strand, the BRCA protein not working is "a catastrophe in the making", states Mukherjee. (Mukherjee, 2016). Damaged DNA means a loss of information and therefore retails in more and more mutations, eventually evolving into breast cancer. Of course, the process might be accelerated or mitigated by certain environmental factors like exposure to X-rays.

Knowing to have a BRCA1 or 2 mutation tells a woman nothing about the point of time or the type of cancer (aggressive and inoperable or therapy-sensitive) she might suffer from. Therefore, a decision what to do with the knowledge of carrying a BRCA1/2 mutation is difficult. One could decide to do nothing and just take things as they come. Another option would be constant screening through for example regular mammograms and MRIs, in order to detect the breast cancer in a primal stadium. Moreover, preventative therapy, such as hormone therapy, can reduce the risk of developing some, but not all types of breast cancer. A rather radical, but efficient possibility to prevent breast cancer is to have a bilateral mastectomy, so removing both of the patient's breasts.

The option to just remove the BRCA mutations in somatic cells, is a rather unrealistic one, because, as already mentioned in 3.3.1., repairing the gene in one copy, does not correct all other cells. However, if the mutated gene was reverted to its original state during a still totipotent[26] stadium, such as the embryonal, the edited version of the gene would be disseminated in every cell of the body.

Hypothetically, the removal of a BRCA mutation would be possible, since the human genome is sufficiently researched. Additionally, BRCA is a single gene mutation, which makes it relatively easy to be cut out. (cf. Mukherjee, 2016)

Also, the risk of an eventual off-target mutation could be kept to a minimum. During the 8-cell stadium one cell can be taken out and checked on, via preimplantation genetic diagnosis[27]. If needed, the cells could be corrected before being planted in the women's uterus. However, it is uncertain whether the embryo would survive repeated genetic modification. (cf. Mündlein, 2019)

Still, the greatest concerns are of ethics and law. There is for example no knowledge about long-term effects after an intervention in the embryonal sta-

[26] totipotent = before cell differentiation; genetic information for all cell types is still activated
[27] preimplantation genetic diagnosis = *also:* PGD (short form); genetic examination of embryos conceived via IVF[28]

dium. Potential side effects would then be passed down to every following generation and nearly impossible to be reversed. (cf. Doudna & Sternberg, 2017) And from a juridical point of view, in Austria such an undertaking would not possible due to the restrictive laws on germline[16] editing. [for further information see 4.2.]

In addition, in this case, there is not really a point in editing the embryos genome. During in vitro fertilization[28] multiple embryos are conceived and via PGD it can be testified which one bears a BRCA mutation. In further consequence, the healthy one can be chosen to be transplanted. (cf. Mündlein, 2019)

[28] in vitro fertilization = *also:* IVF (short form); artificial insemination "in a culture dish"

4. Ethical tenability and legal provision

A scientific innovation usually is only brought to common application after having passed a series of checkpoints. One of those points is the question whether or not it is considered ethically tenable to proceed. This is an issue of discussion that needs to be revised over and over again, especially with such a powerful device like CRISPR/Cas9. (cf. Rodriguez, 2016) Due to those ethical issues in some countries, like Austria, restrictions on gene therapy are strict and do not allow interference in the germline[16]. (cf. RIS, 2019)

4.1. Risks of CRISPR/Cas9 and ethical conflicts

With CRISPR/Cas9 scientists now have a new tool for genetic engineering that provides a lot of benefits. It simplifies the editing process, has high fidelity and lacks immense costs. In online shops like Synthego, one can already order "CRISPR kits", ready to work with. (cf. Synthego, 2018) The danger of every amateur scientist being able to modify cells is nevertheless not given, opines Mündlein. CRISPR/Cas9 and its adequate use is still limited to well-equipped research labs. (cf. Mündlein, 2019)

The actual problem is that unless being regulated, it can be not only be used for therapeutic reasons but basically for any purpose. In theory, individual non-health enhancements like e.g. a higher performance possibility for athletes lie in the range of possibilities.

In addition, nearly all types of cells can be targeted by CRISPR/Cas9, which includes not only somatic, but also germ cells. As alterations in the germline[16] are heritable, any change, and therewith mistake, would affect all further generations. (cf. Rodriguez, 2016)

4.1.1. Editing somatic cells

Generally, in scientific research it is essential to balance the benefits and risks of what one is examining. Only if the advantages clearly overweigh the risks it is ethically correct to proceed.

Using CRISPR/Cas9 the main risk is that a change to the genome may produce off-target effects. [for further information see 2.3.] The cleavage of those similar but not intentionally targeted sequences evidently can turn out problematic and lead to mutations or even cell death. To evaluate the risks, it is thus necessary to be able to estimate the frequency of off-target effects.

A factor needed to be considered when talking about CRISPR/Cas9 applications in humans is that the laboratory research is often conducted in mice or zebrafish, whose genomes are quite small. When dealing with the larger and much more complex human genome, the probability that similar sequences and therewith off-target effects might occur is higher. (cf. Rodriguez, 2016)

One also needs to distinguish between an *in vivo*– and an *ex vivo*–intervention. Intervening *in vivo,* so for example inserting a CRISPR/Cas9 complex directly into the patient's body and letting it edit the genome onsite, is riskier. Once the modifications, including any off-target changes, are made, it is very difficult to reverse them. And not only are those interventions permanent, but they will also be in the genetic material of any new cell descending from the original one. Contrarily, in an *ex vivo* approach, cells are taken from the patient, edited in the laboratory and then inserted back into the body. In this way, it is still possible to check on the edited DNA and, in case anything went wrong, to resurrect it before going any further. (cf. Doudna & Sternberg, 2017)

Another thing one has to take into account is that the effects resulting of editing DNA are not always predictable. A little change can either barely make a difference or affect the genome on several layers. Mukherjee compares this whole process to a recipe.

> "[…] the alteration of a recipe or formula does not change the product in a predictable manner: if you quadruple the amount of butter in a cake, the eventual effect is more complicated than just a quadruply buttered cake, […] [it] collapses in an oily mess." (Mukherjee, 2016)

In addition, the genes themselves do not necessarily interact systematically. Mostly, they react to their environment and are randomly occurring. It is thus rather difficult to say with certainty what modifications to the genome will result in eventually.

In the end, there is also the question when to intervene. Geneticists normally stay within the boundaries of three moral lines. This triangle consists of the keywords highly penetrant mutations, extraordinary suffering and justifiable interventions. An example for that would be to test for a mutation that will with a probability of almost 100 percent result in cystic fibrosis[29]. Those are also usually illnesses that make it nearly impossible to lead a "normal" life. It is then, up until now, the choice of every individual whether or not he/she wants to interfere. This guideline for accepted interventions has been defined by the ethical understanding of the society. Moral consensus is thus also let to decide what are "justifiable interventions" and whether or not they are limited to therapeutic necessity. (cf. Mukherjee, 2016)

4.1.2. Editing germ cells

Germ cells are the cells making up the germline[16], so those that are inherited by the progeny. That includes next to sperm and egg cells also embryonic cells in a still totipotent[28] stadium, which are often referred to as ES cells ("embryonic stem cells").

The advantage and at the same time disadvantage of germ cells is that one can edit a single cell and this will have effect on the whole developing organism.

[29] cystic fibrosis = disease condition caused by a point mutation on the CFTR gene; leads to slime accumulation in the lungs

When wanting to erase an error in the genome it is impossible to do so in somatic cells, because really getting rid of it would mean to modify every cell in the body. Contrarily, after editing a germ cell the modification will be found in every descending cell. Naturally, this is the same for non-intended changes. (cf. Doudna & Sternberg, 2017)

More importantly, however, is the fact that any changes in germ cells will be passed down to the progeny. Which again, could be an advantage, as for example a mutation supporting a genetic disease can get erased once and for all. Nevertheless, also defaults will be inherited. As germ cells would be edited after IVF[28] one could argue that, since it would be *ex vivo*, off-target effects can be ruled out by intense screening before inserting the fertilized cell. However, there are no long-term studies about possible side effects that might occur afterwards and would affect all further generations. (cf. Rodriguez, 2016)

Furthermore, allowing *ex vivo* germ cell editing could open a door for eugenics. This does not necessarily mean to start editing embryo cells for "enhancement", like better genetic preconditions for practicing sports or a hair colour of choice. But it basically provides the possibility to select foetuses without aborting them. Parents or doctors have to make a decision, declaring one as the correct and perfect embryo and at the same time downgrading the others. For example, simply having to choose between a healthy male and healthy female embryo gives one sex another value than the other. (cf. Mukherjee, 2016)

But generally, it is to ask if CRISPR/Cas9 will be used in such a field at all. Back in 2015 scientists like Lanphier and his colleagues did not consider this technique ready for germline application. (cf. Lanphier, et al., 2015) Discoverer Jennifer Doudna stated that the question to pose is not if germ editing will eventually happen, but rather where and when. She is therefore encouraging ethical discussion about how to best handle this new tool. (cf. Doudna & Sternberg, 2017) General opinion is that the method should be "fully safe" before risking any therapeutic

application in the germline. If it was, eventually, further discussion would be necessary, taking into account social, legal and ethical standards as well as the need for regulatory laws in order to prevent abuse.

Back in 1997 the Universal Declaration on the Human Genome and Human Rights was declared by the UNESCO, calling for a moratorium for germline interference. During the International Summit on Human Gene Editing held in 2015, however, the participating members of scientific academies of various countries agreed to continue with basic and clinical research with embryotic cells. Nevertheless, every country has its own regulations. In Great Britain for example embryonic engineering is lawfully approved, if only for research, whereas in Austria any intervention in the germline is prohibited. [for further information see 4.2.] Heritable changes were generally considered as irresponsible by the Summit. Moreover, they founded an international forum where ethical concerns can be ongoingly discussed. (cf. Rodriguez, 2016)

An example for a recent subject of discussion would be the newly-born Chinese twins Nana and Lulu. Doudna seemed to be right about her statement mentioned earlier, because the team around He Jiankui from the University of Shenzhen engineered embryotic cells and planted them back into the womb of the mother. The aim was to make the children HIV-resistant. The scientific community is bewildered by this complete ignorance of ethical standards and discussions about how to handle this situation are ongoing. (cf. Irmer, 2018)

The effects of this interference will get visible in the upcoming years. And this brings along the question: “What if anything went wrong?”. It is to be considered that every single change that was made will be passed down to their progeny if Lulu and Nana want to have children. And who is the one to blame, if there then is a falsely edited gene in their genome.

4.2. Possibility of interference with CRISPR/Cas9 in Austria

4.2.1. Law for genetic engineering

Before being able to apply gene therapy, information needs to be collected by analysing the patient's genome. Referring to §65 of the Austrian Law for genetic engineering a genetic analysis is only to be conducted by the current state of scientific and technological knowledge and can be divided in four types. One speaks of type 1 if the aim is to prepare or control a therapy method that is based on knowledge about somatic modifications against a currently occurring illness. If the disease is the result of a germline[16] mutation, it is declared to be type 2. Genetic analyses of type 3 and 4 seek to detect a predisposition, especially a genetic one. The possibility of treating or preventing it makes it a type 3, whereas the lack of possible therapy or prevention entails type 4.

The data gained in the process can be only used for research with anonymous samples.

Before a genetic analysis can get started each case needs to be individually admitted. In addition, the head of the executing laboratory needs to be able to show several qualifications and at least two years of practical experience in this field of technology.

The next step, somatic gene therapy, will only be conducted in two cases (§74). Either to prevent or give therapy to severe diseases or in order to find a cure for those illnesses due to clinical trials. Furthermore, it needs to be assured that there is no risk of inducing modifications to the germline. If a change to the heritable genetic material cannot be ruled out, the gene therapy is only admitted if the expected benefits clearly overweigh the risks and if the patient will certainly not have any progeny. When all those criteria are fulfilled an ethics committee will review the case and in further consequence admit or defeat it. (cf. RIS, 2019)

4.2.2. Law for reproductive medicine

In Austria, in vitro fertilization[28] is only admissible in a marriage or a registered partnership. It can be conducted when every other method to conceive a child failed or sexual interference isn't an option due to possible risk of communicating an infectious disease or both partners having the same sex.

The conceived embryo will further solely be checked on via preimplantation genetic diagnosis[27] if one failed to induce a pregnancy via IVF for three or more times or the woman experienced a minimum of three miscarriages or stillborn children, which, in each case, needs to be supposedly linked to a genetic disposition. Another reason to examine the embryo is a genetic disposition in the parents' genome which entails the danger of experiencing a miscarriage or a stillborn child. PGD can also take place if a genetical disease exists in the family that can lead to severe restrictions which make it impossible to lead an autonomous life.

The only admissible aim of examining embryos via PGD is to avoid the occurrence of those severe restrictions. This also means that stem cells can solely be used for medically supported reproduction, so in order to induce pregnancy or for PGD. Intentional germline[16] interference is unexceptionally prohibited by Austrian law. (cf. RIS, 2018)

4.2.3. Questioning the Austrian population on CRISPR/Cas9

On occasion of the recent events in China, when two babies, prior edited with CRISPR/Cas9, were born, the "market Institut" in Linz, Austria conducted a survey on Austria's attitude towards CRISPR/Cas9 and genome editing in general.

The amount of people that are actually educated about the topic "genetic manipulation in humans" is rather small. Contrarily, the percentage of those who are little or not at all informed about genome editing makes up more than two thirds of the sampled population. [see Figure 7]

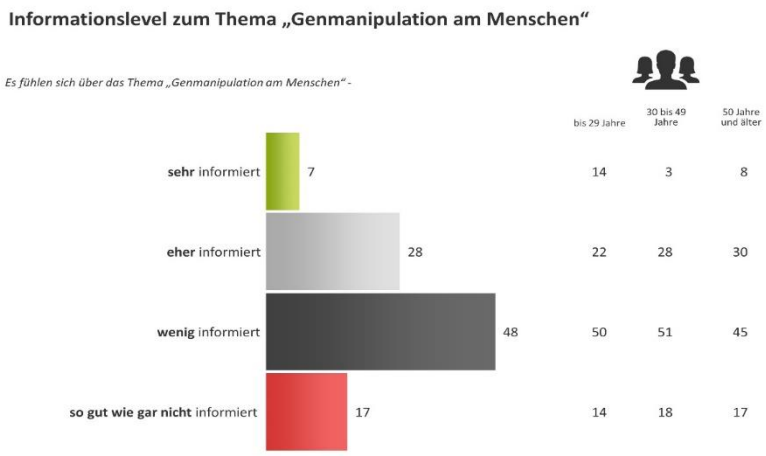


Figure 7: Information status of the Austrian population on genome editing (Market Institut, 2018)

Conclusively, there is a lot of scepticism concerning gene manipulation. In accordance with the findings of the "market Institut" only about 10 per cent see genome editing in a positive way. The rest either feels rather uncomfortable when imagining it or is of the opinion that, in general, there should be no tool for "playing God". [see Figure 8] (cf. Market Institut, 2018)

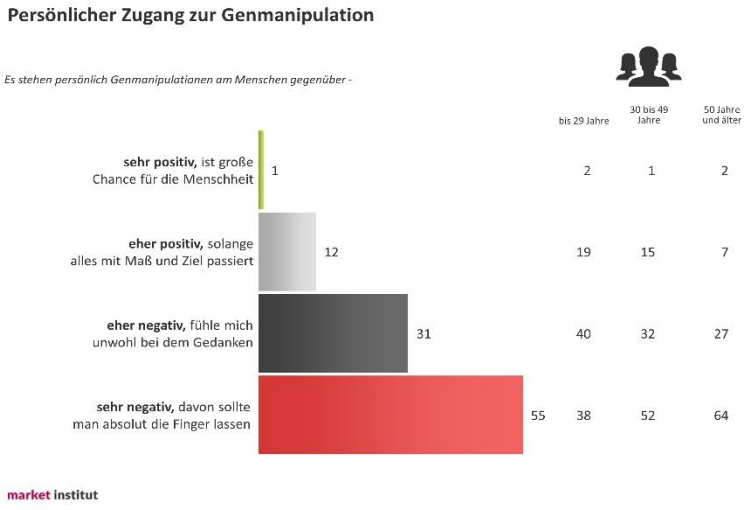


Figure 8: Opinion of the Austrian population on genome editing (Market Institut, 2018)

5. Summary and Outlook

CRISPR/Cas9 provides researchers with a unique tool for genetic engineering. Apart from its main drawback, off-target effects, it can be used simply, inexpensively and efficiently for both genome editing and screening. Therefore, it supports basic research on breast cancer and helps to invent new strategies that specifically target certain cancer hallmarks.

However, once the cancer breaks out, there is little sense in trying to erase all the cancer driving mutations as CRISPR/Cas9 is often not qualified to fight the too complex tumour exome. Therefore, the great potential rather lies in fighting resistance against already existing methods or in enhancing them. As researchers now have a tool to edit the epigenome in a more targeted fashion, experts see high chances of success in avoiding side effects of epigenetic treatments of breast cancer. Furthermore, certain modifying processes can be simplified and individualized by using CRISPR/Cas9, which enables advances in immunotherapy.

Still, all the trials harnessing CRISPR for therapeutic use in somatic cells are in their beginnings. Clinical trials investigating the safety of applying CRISPR/Cas9 to the human body need to be conducted first. This means that it is not until the upcoming years that the different approaches will prove themselves suitable for broader application.

Then again, the situation is different when it comes to germline alterations. When wanting to erase inherited BRCA mutations, an intervention would be of unnecessary risk. In addition, it is generally ethically questionable to edit germ cells. At the moment, this topic is wildly discussed. The ethical consensus of only allowing germline editing for research could be changed, as the chief of the WHO recently summoned an expert committee and does not want to rule out therapeutic germline interference beforehand. (cf. APA, 2018)

Whatever the case will be, at the current state of affairs, CRISPR/Cas9 supported gene therapy would have a lot of difficulties in Austria. Not only are the possibilities of applying somatic gene therapy to patients limited due to law restrictions, but also the current attitude of the Austrian population towards this topic could be an inhibitory factor. Logically, there is little sense in inventing new treatment methods that are then rejected by the society. It is therefore essential to educate a broader mass about both the risks and the immense potential of CRISPR/Cas9.

Glossary

[1] archaea = *also:* archaebacteria; prokaryotic organisms (cf. Spektrum Akademischer Verlag, 1999)

[2] RNA = nucleic acid; encodes genetic information; similar to DNA, but single-stranded (cf. Spektrum Akademischer Verlag, 1999)

[3] nucleic acids = hypernym for DNA and RNA, built of "nucleotides" (= phosphate residue + [deoxy]ribose + base) (cf. Spektrum Akademischer Verlag, 1999)

[4] spacer sequence = piece of viral DNA that was embedded in the bacterial genome (cf. Max-Planck-Gesellschaft, 2018)

[5] prokaryotes = organisms whose cells do not have a nucleus; *opposite:* eukaryotes (cf. Spektrum Akademischer Verlag, 1999)

[6] eukaryotes = organisms whose cells do have a nucleus; *opposite:* prokaryotes (cf. Spektrum Akademischer Verlag, 1999)

[7] double-stranded break = *also:* DSB (short form); both DNA strands get cut simultaneously (cf. Spektrum Akademischer Verlag, 1999)

[8] phage = *also:* bacteriophage; virus that infects bacteria and archaea to propagate in its host (cf. Spektrum Akademischer Verlag, 1999)

[9] bases = *more specifically:* nucleic acid bases; components of DNA and RNA; always bind to their complementary base (Adenine – Thymine/Uracil; Cytosine – Guanin) (cf. Spektrum Akademischer Verlag, 1999)

[10] hybridisation = process when two complementary nucleic acid strands congregate to a double strand (cf. Spektrum Akademischer Verlag, 1999)

[11] TALEN = "transcription activator-like effector nuclease", artificial protein used for genome editing (cf. Bortesi & Fischer, 2015)

[12] ZFN = "zinc finger nuclease", artificial protein used for genome editing (cf. Bortesi & Fischer, 2015)

[13] methylation = annealing of one or several methyl group(s); methylation of the DNA is responsible for cell development and cellular differentiation (cf. Spektrum Akademischer Verlag, 1999)

[14] epigenome = genetic information that cannot be ascribed to the actual DNA; epigenetic changes = heritable changes in gene expression due to environmental impacts; (in)activation of certain genes through e.g. methylation (cf. Spektrum Akademischer Verlag, 1999)

[15] point mutation = one base in the genetic code is exchanged, added or deleted (cf. Spektrum Akademischer Verlag, 1999)

[16] germline = evolution of a fertilized egg cell to germ cells (=sexual cells) (cf. Spektrum Akademischer Verlag, 1999)

[17] HER2 = “human epidermal growth factor receptor 2”; oncogene that stimulates cell proliferation (cf. Kingston & Johnston, 2016)

[18] chronic radiation pneumonitis = disease condition caused by changes to the lung tissue resulting in constant shortness of breath (cf. Cancer Research UK, 2017)

[19] pulmonary fibrosis = disease condition caused by irreversible scarring of the lung tissue; often result of chronic radiation pneumonitis (cf. Cancer Research UK, 2017)

[20] osteoporosis = weakening of the bones, which e.g. increases the risk of having bone fractures (cf. Cancer Research UK, 2017)

[21] monoclonal = *here:* only one type of antibodies is used (cf. Cancer Research UK, 2017)

[22] cyclin = protein that has impact on the regulation of the cell cycle (cf. Spektrum Akademischer Verlag, 1999)

[23] apoptosis = controlled, self-induced cell death (cf. Spektrum Akademischer Verlag, 1999)

[24] T-cell = cells of the immune response; responsible for adaptive immunity (cf. Spektrum Akademischer Verlag, 1999)

[25] non-coding RNA = does not encode proteins, but can control gene expression (cf. Khan, et al., 2016)

[26] totipotent = before cell differentiation; genetic information for all cell types is still activated (cf. Spektrum Akademischer Verlag, 1999)

[27] preimplantation genetic diagnosis = also: PGD (short form); genetic examination of embryos conceived via IVF (cf. Spektrum Akademischer Verlag, 1999)

[28] in vitro fertilization = also: IVF (short form); artificial insemination “in a culture dish” (cf. Spektrum Akademischer Verlag, 1999)

[29] cystic fibrosis = disease condition caused by a point mutation on the CFTR gene; leads to slime accumulation in the lungs (cf. Spektrum Akademischer Verlag, 1999)

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Table of Figures

Appendix

Interview with Dr. Axel Mündlein from the VIVIT Institute in Dornbirn/Austria (15.01.2019):

1. *CRISPR/Cas9 has a lot of potential for modern medicine. What would you say are its greatest advantages, of which application can one benefit the most?*

   Now: basic research, so understanding the functional role of genes; the development of genetic modified plants (this is hampered in the EU; *see: "EUGH-Entscheidung zur Genomeditierung in Pflanzen"*). In the future: cure of hereditary diseases or cancer.

2. *On the other hand, especially with nearly every amateur scientist being able to have access to "CRISPR kits" nowadays, which do you think are its greatest dangers?*

   Although everybody may order "CRISPR kits". However, the question is, if the kits can be used adequately. In my opinion, the CRISPR/Cas9 method is restricted to experienced researchers in well-equipped laboratories.
   One of its greatest dangers may be the facilitated production and release of GMOs (particularly plants) in countries without legal regulations or generally accepted ethical standards, which may be spread around the world.

3. *In order to use CRISPR/Cas9 as a gene editing tool in an efficient way, there are some requirements. What would you consider necessary?*

   At least, laboratory equipment for working with cell cultures and for DNA analysis. For a more detailed answer please ask somebody, who is using CRISPR/Cas9 practically.

4. *When wanting to target a specific mutation, knowledge about the targeted DNA sequence is required. Would you consider breast cancer associated genes sufficiently researched to build an exact gRNA?*

   Yes: gRNA is short and human whole genome sequence is known due to large sequencing projects started in the last century. The same is true for most domestic organisms.

5. *Where do you see difficulties when wanting to erase all cancer-supporting/-inducing mutations?*

   Using next generation sequencing technologies, the whole genome, or at least the whole exome of the tumour can be sequenced at moderate costs. However, this is only true for labs with high end next generation sequencing technologies (which, to my knowledge, are rare in Austria). However, at least theoretically, there are no limitations in mutation detection. However, I don't know if it is possible to erase mutations in different genes simultaneously.
   It should be further noted, that 1. The effect of a detected mutation on tumour development or progression is often unclear; most mutations are passenger mutations and not driver mutations; 2. tumours can be polyclonal; that means that a tumour may be composed of cells from at least two distinct progenitors with different mutational profiles. And 3. not only gene mutations but also epigenetic changes or gene copy number changes are involved in the development of cancer. E.g. the amplification of EMSY or hypermethylation of Rad51C also leads to BRCA silencing and the development of breast or ovarian cancer. *(see: Rigakos et al., 2012: BRCAness: Finding the Achilles Heel in Ovarian Cancer. THE ONCOLOGIST Express)*.

6. *Could BRCA1/2 genes be erased in a still totipotent stadium, is the knowledge sufficient?*

   Theoretically yes, because CRISPR/Cas9 has been successfully used in studies on embryo development *(see: Fogarty, N. M. E. et al. Nature http://dx.doi.org/10.1038/nature24033 (2017))*.

7. *Hypothetically: if one wanted to erase the BRCA mutation in an embryotic cell, that would be ex vivo. So, via pre-implantation diagnostics, one could check if anything went wrong and further correct it?*

   At 8 cell stage, one cell can be removed and genetically analysed. The missing cell will be replaced, and a viable embryo may be transplanted. This is state of the art in pre-implantation diagnostics (but strictly forbidden in Austria and other EU countries). Therefore, it appears possible, to check the result of genome editing. However, it is questionable if the embryo will survive further genome editing. Also, the sense of genome editing in case of BRCA mutations is questionable. Using in vitro fertilization several embryos can be produced which can be genetically analysed. One of the embryos without a BRCA mutation can be transplanted (not allowed in Austria). This has been practised in the UK 10 years ago *(see: https://www.theguardian.com/science/2009/jan/09/breast-cancer-gene-baby)*.

8. *In a lung cancer trial, they cut out the gene that encodes PD-1 receptors by using CRISPR/Cas9. A new approach in treating triple-negative breast cancer is to keep PD-1 from inhibiting the immune response in tumour cells. In theory: as PD-1 is erased, could that work for TNBC too?*

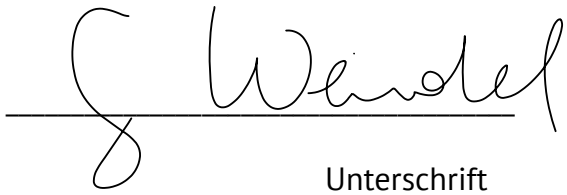
   To my knowledge, only safety and not immune response of PD-1- T lymphocytes therapy in lung cancer patients has been evaluated in a phase I trial. *(see: http://cancerres.aacrjournals.org/content/78/13_Supplement/CT133; http://ascopubs.org/doi/abs/10.1200/JCO.2018.36.15_suppl.3050)*
   If PD-1-/- T therapy will improve survival in NSCLC patients, the therapy may be applied in other cancer patients sensitive for immunotherapy strategies. However, ongoing and future trials have still to define the role of immune checkpoint blockade in the treatment of TNBC. *(see: https://www.go-toper.com/publications/ajho/2017/2017may/immunotherapy-for-triple-negative-breast-cancer-a-focus-on-immuno-checkpoint-inhibitors)*

Selbstständigkeitserklärung

Name: Stephanie Wendel

Ich erkläre, dass ich diese vorwissenschaftliche Arbeit eigenständig angefertigt und nur die im Literaturverzeichnis angeführten Quellen und Hilfsmittel benutzt habe.

Feldkirch, 20.02.19



Unterschrift