Katherine Blix

Biomedical engineering state-of-the-art and future perspectives: Three novel editing tools' potential in translational medicine for noncommunicable diseases

Master's thesis in Molecular Medicine

Trondheim, June 2021

Supervisors:
Nadra J. Nilsen and Erlend B. Tande

Norwegian University of Science and Technology

Faculty of Medicine and Health Sciences

Department of Clinical and Molecular Medicine

NTNU

Norwegian University of Science and Technology

ABSTRACT

Biomedical engineering has seen great progress since the development of new gene-editing and RNA-editing technologies. At the moment, there are numerous ongoing biomedical research that utilize new technologies for editing DNA or RNA.

This thesis aims to investigate the potential of three novel biomedical engineering editing tools (namely CRISPR/Cas9, prime editing, and programmed RNA-editing) in translational medicine for noncommunicable diseases. Different studies have been taken into consideration, and independently examined based on *in vivo* (or similar tests, i.e. tests done on organoids) and preclinical trial studies. Moreover, the most recent knowledge on chronic inflammation is given as a basis to understand noncommunicable diseases. In addition, future perspectives on the use of these editing tools in basic biomedical research for these diseases are discussed. An experiment model is also presented as an example of designing CRISPR/Cas9 gene target guides for knockout of the scavenger receptor CD36 to study atherosclerosis.

The results gathered from the literature review on the three novel editing tools have highlighted the lack of knowledge that there still is on prime editing and programmed RNA-editing tools, and the need to further investigate their potential in translational medicine. Overall, this thesis highlights how each of the tools taken into consideration hold its own unique potential in translational medicine, if used for studying or treating different specific diseases. Therefore, since all three biomedical engineering editing tools mentioned herein seem to have all great potential in transcriptional medicine, they should not be seen as a good or worse alternative to each other, but rather different solutions for different diseases.

ACKNOWLEDGEMENTS

This thesis is a part of the MSc in Molecular Medicine study programme in the Faculty of Medicine and Health Sciences (MH) at the Norwegian University of Science and Technology (NTNU) in Trondheim, Norway.

I would like to thank my supervisors Nadra Jesmine Nilsen, and Erlend Bjørkøy Tande for their incredible patience and support throughout this project. In particular, I want to express my gratitude for having been given this incredible opportunity to write such an unconventional master thesis. Thank you for your continuous precious guidance and advice during the writing process, and for all the lessons and interesting discussions.

I am also grateful to my student advisor Marit Barstad for finding a way to accommodate my needs which did not allow me to carry on my work physically at the university laboratory. Thank you for making it possible for me to get my master despite my struggles.

Finally, I want to thank Victor Boyartchuk for his valuable insights which helped me to get more familiar with the CRISPR/Cas9 system experimental methods.

TABLE OF CONTENTS

1. INTRODUCTION

Technological progress has brought humanity incredible scientific advancements, above all in the field of medicine. This has given us the opportunity to increase human life's quality and life expectancy. However, as the quality of life and global average age increases, so does the incidence of chronic diseases, best known as *noncommunicable diseases*, i.e. non-infectious diseases (e.g. cardiovascular diseases, diabetes, cancer etc.) (World Health Organization, 2018). The increase in the average age of the population also increases the risk of developing chronic inflammation. Low-grade chronic systemic inflammation has been shown to be associated with most noncommunicable diseases (Philips et al., 2019).

The increased risk of developing low-grade inflammation is due to different factors including unhealthy diet (e.g. high trans-fatty acids intake) and microbe dysbiosis; sedentary lifestyle; social and physical environments; and lastly, different environmental and industrial toxicants exposure (Furman et al., 2019). Furthermore, longer life expectancy results in an increase of individual risk to immune dysregulations associated with aging; the most relevant being the high levels of pro-inflammatory biomarkers in the circulation (Ferrucci and Fabbri, 2018). The more research is done on the topic, the more the link between chronic inflammation and age is becoming clear. In fact, chronic inflammation is observed so frequently in older organisms that in 2000 an Italian Prof. Emeritus of immunology, Claudio Franceschi, coined the term "*inflamm-aging*" (Franceschi et al., 2000). Genetic susceptibility has been shown to also be a possible risk factor not only of inflammaging (Ferrucci and Fabbri, 2018) but also of chronic inflammation in different population ages (Nasef et al., 2017).

Noncommunicable diseases incidence increases by the year (CDC.gov), yet the way we understand and treat them have not taken the urgency that should have been taken decades ago. Despite the known correlation between technological advancement, the increase of the general population's age and noncommunicable diseases, we do not yet know how to cure these diseases. This is mostly due to an intrinsic lack of understanding of how (and why) these diseases affect each singular individual.

As new technologies are developed under the pressure of modern progress, it becomes clearer that the approach on noncommunicable disease treatments will have to increasingly become

more tailored to the individual patient's unique clinical picture. However, before we are able to do this, we first and foremost must know what solutions are available to us to better understand these diseases, and thus later find better treatments.

The COVID-19 crisis has reminded us how chronic illnesses and their current treatments weaken the general population's health, making it more exposed to the unpredictability of modern health challenges. Therefore, it is of paramount importance to learn more of the most current novel technologies that could have great potential to give better treatments in medicine and thus become future solutions to these health challenges.

This thesis attempts to fill this gap by exploring what are the most current technologies (focusing in particular on biomedical engineering editing tools), what is currently known about inflammation and its role in communicable diseases, and what are these tools' most promising applications for noncommunicable diseases research and treatment. Some future perspectives on the matter will also be given at the end of the thesis.

1.1. Genetic engineering: From the discovery of DNA to the current cutting-edge genetic and post-transcriptional engineering editing methods

The scientific advances of the last decades have made it possible for us to better understand genetics and molecular biology. This in turn has led to the unravelling of more and more details of the structure and functions of the genome.
Although Deoxyribonucleic Acid (DNA) was discovered in 1869 by Friedrich Miescher, and then characterized later in 1953 by Watson and Crick (Watson and Crick, 1953), it was only at the end of the 1960s that genetic engineering started to rise as a new scientific technique.

What is meant by genetic engineering and what scientific progress led to it
Genetic engineering is defined as the process by which it is possible to design and genetically modify an organism's DNA using biotechnology. Through genetic engineering of an organism, scientists are able to combine DNA from different sources. This method is also known as *recombinant DNA technology*. Thanks to this technique it was possible to produce many proteins of medical importance, like insulin (Thieman and Palladino, 2014). The very first example of the application of this method was done in 1972 by Paul Berg, a biochemist who was able to produce restriction enzymes to perform DNA recombination. Together with his colleagues, Jackson and Symons (Jackson et al., 1972), they developed methods for covalently joining duplex DNA molecules to construct circular dimers to insert a DNA segment into a polyomavirus (the Simian virus 40). The revolutionary potential of this new technology opened the door to improve genetic studies on human genetic diseases (among other industrial applications), which culminated with the starting of the *Human Genome Project* in 1990.

The possibility of disruption of different genes has made it doable to characterize and map many different genes based on the phenotypic effects the disruptions had on the modified organism. Biomedical engineering has progressed enormously thanks to this new recombinant technology. Above all in the last decades, there has been a quick evolution of innovative approaches. From the early site-specific recognition of DNA sequences, to the site-directed zinc finger nucleases (ZNFs); to then the DNA protein recognition of TAL (transcription activator-like) effector nucleases (TALENs), to the recent clustered regularly interspaced

short palindromic repeats (CRISPR) and Cas9 associated protein; and finally to the most recent "prime editing" method reported by Anzalone et al. in 2019 (Anzalone et al., 2019).

CRISPR/Cas9

The CRISPR/Cas9 system is an efficient gene-editing tool for editing genomes of human cells. This system was originally observed in *Escherichia coli* bacteria, and it is characterized by a series of short direct repeats interspaced with short sequences in the genome, hence the name "clustered regularly interspersed palindromic repeats" (CRISPR).
It was hypothesized that these sequences played an important role in DNA repair or gene regulation for the adaptive immune system of certain bacteria and archaea. This hypothesis was then confirmed when it was observed that the CRISPR-associated *cas* genes encode proteins with putative nuclease and helicase domains. The first experimental evidence of CRISPR/Cas9 as an adaptive defence system, was provided by infection experiments using *Streptococcus thermophilus* bacteria. The findings of these experiments lead to the very first biotechnology use of the system, which was harnessed for immunization against phages in the dairy industry. The targeting activity of the system was later reported thanks to experiments performed in the pathogen *Staphylococcus epidermidis* (Jinek et al., 2012; Doudna and Charpentier, 2014) (for more insights on how CRISPR/Cas9 works, see subchapter 4.1.1.).

Prime editing

Prime editing (PE) is a further expansion of what is possible to achieve using the CRISPR/Cas9 editing tool. The PE method uses exogenous reverse transcriptase activity. Therefore, the genomic DNA is edited by being "overwritten" instead of edited out. This new Cas-based system has been developed by Liu et al. (Anzalone et al., 2019) in collaboration between the Broad Institute of MIT and Harvard, US (for more insights on how prime editing works, see subchapter 4.1.2.)

Beyond CRISPR/Cas9 gene-editing: The development of programmed RNA-editing

As of late, more interest has been slightly shifted towards programmed Ribonucleic Acid (RNA) editing as well, which allows for a more "down the line" editing. This type of programmed editing method, developed by the researchers Stafforst and Schneider, involves the editing of messenger RNAs (mRNAs) instead of the DNA sequence from which the protein of interest derives from (Stafforst and Schneider, 2012). Regrettably, the timing of the development of this last approach was quite unfortunate, since it came about the same time as

the publishing of the study on the DNA-editing tool CRISPR/Cas9 led by Dounda and Charpentier, with the contribution of the biochemist Martin Jinek (Jinek et al., 2012).
The study on mRNA-editing by Stafforst and Schneider (Stafforst and Schneider, 2012) was overshadowed by the revolutionary aspects of CRISPR/Cas9 which is relatively easy to use, unique in its DNA cleaving mechanisms (including its multiplex target recognition never seen before) and cost-effective. At the time, it seemed almost useless to edit mRNA when there was already available such an advanced novel DNA editing tool as CRISPR/Cas9, which not only promised a "precise and efficient targeting, edit, modification, and regulation, but also the marking of genomic loci of a wide array of cells and organisms" (Doudna and Charpentier, 2014) (for more insights on how programmed RNA-editing works, see subchapter 4.1.3.)

1.2. Biomedical engineering takes on genetic engineering methods: The two most recent examples of pre-clinical applications

Whilst in the past the medical use of genetic engineering was mainly considered relevant for pharmaceutical manufacturing and for gene replacement therapy, its use is now becoming more and more eclectic. The editing tools taken into consideration in this thesis are an example of such eclecticity.
Just in the year 2020, the potentiality of each of these gene-editing tools has appeared to be astonishing. In this section there are briefly reported the most important breakthroughs in the year 2020 for each genome editing tool (namely CRISPR/Cas9 and prime editing), just as examples of the giant leaps genetic engineering is taking in biomedical engineering.

CRISPR/Cas9 has been shown to be able to silence the gene involved in sickle-cell disease, but targeting the mRNA instead of the gene was also shown to be a valid alternative
CRISPR/Cas9 technology was recently used for developing a method to treat the root cause of sickle-cell anaemia, attributable to the polymerization of deoxygenated sickle hemoglobin (HbS) (Frangoul et al., 2020; Esrick et al., 2020).

Two studies have recently been published that present two different ways to treat sickle-cell disease using two different methods; one involving CRISPR/Cas9 editing of the gene involved in the disease the *BCL11A* (B-cell lymphoma/leukemia 11A) gene, and the other involving the disruption of the protein transcripted by the same gene.
The first study reported favorable results in targeting the *BCL11A* erythroid-specific enhancer. The second study presented the targeting and silencing of the *BCL11A* mRNA.
Both studies show promising results, but present limitations, which are great enough for not allowing for a favorable assessment as possible future long-term treatments. However, the second study represents an important step towards possible alternatives to the more permanent CRISPR/Cas9-based experimental treatments. In fact, targeting the mRNA instead of the gene itself alters only the genetic expression and does not modify the original coding sequence (Esrick et al., 2020); this results in a better control on possible side-effects of the genetic intervention, thus presenting no risk for the rest of the genetic material in the cells. This aspect in particular allows for better prediction and characterization of side-effects, which in turn can lead to a better assessment of the cost/benefits of the treatment itself. This last aspect is at the base of what is observed when using RNA-directed editing, as it will be explored later on (for more insights, see subchapter 4.1.3. and 5.1.4.).

Prime editing has shown to be promising in treating genetic defects

In a recent study (Schene et al., 2020) the prime editing tool was used for editing primary adult stem cells derived from *in vitro* growing of organoid culture models. In the study there was shown that the prime editing method was not only effective and accurate to create mutations in organoids that mimicked human cancer cell lines, but also to correct disease-causing mutations. Moreover, the study shows that the prime editing does not induce genome-wide off-target effects (Schene et al., 2020) (for more insights, see subchapter 4.1.2. and 5.1.2.)

1.3. Translational medicine and the link between chronic inflammation and noncommunicable diseases

What is meant with translational medicine

Since this thesis aims to explore the aforementioned biomedical engineering editing tools' potential in translational medicine, it is worth specifying what is meant by "translational medicine (TM)". Herein, the term is defined based on the European Society for Translational Medicine (EUSTM) description of TM, i.e.:

"(TM) is an interdisciplinary branch of the biomedical field supported by three main pillars: benchside (basic research), bedside and community. The goal of TM is to combine disciplines, resources, expertise, and techniques within these pillars to promote enhancements in prevention, diagnosis, and therapies [...] in order to improve the global healthcare system significantly." (Cohrs et al., 2015)

Biomedical engineering editing tools are promising technologies that could further support the interdisciplinary effort of TM to promote enhancements in prevention, diagnosis and novel therapies. This is why it would be valuable to explore if these tools could really help in achieving the goal of TM, and how and in what capacity they can do so. It is possible to do so by determining what potential they actually hold in TM. As it will be attempted to do so in this thesis.

1.3.1. The inflammation process in brief

Inflammation is a physiological immune response of the human body, usually against pathogens, like bacteria and viruses. Thanks to this immune response, the body is also able to protect its tissues and system from toxins. In addition, inflammation promotes tissue repair and recovery (Netea et al., 2017). In some cases, the inflammatory process is triggered also in the absence of pathogenic invasion. Such a response is referred to as sterile *inflammation* (Zindel and Kubes, 2020). During the inflammatory response, numerous immune cells called leukocytes (white blood cells) are recruited on the site of sterile injury. During this phase the inflammatory response is acute. If the inflammation cause (e.g. tissue injury) persists, the acute response is never resolved, thus becoming chronic. This can lead to the establishment of what is called *chronic inflammation*, which stimulates a perpetual leukocyte-mediated

response. This response tries to clear damaged tissue and engage tissue repair in order to restore tissue homeostasis.
The increase of tissue damage triggers the release of Damage-associated molecular patterns (DAMPs). DAMPs can also be triggered by cell stress or injury (Roh and Sohn, 2018) (e.g. cell injury induced by cholesterol crystals, which play an important role in the inflammatory process in atherosclerosis). The DAMPs can be recognized by different types of pattern recognition receptors (PRRs) (e.g. Toll-like receptors (TLRs) and cytoplasmic Nod-like receptors (NLRs)) and also by non-PRRs found in different types of cells.
Upon ligation of DAMPs to receptors on immune sentinel cells, there is a release of pro-inflammatory cytokines and coagulation factors which activate platelets to ensure hemostasis. Platelets themselves carry PRRs, such as TLR2 and TLR4 which can recognize circulating DAMPs, thus contributing to the inflammatory response by releasing cytokines, chemokines, and other inflammatory mediators (Zindel and Kubes, 2020).
Ultimately, the DAMPs release leads to enhanced leukocyte recruitment; this, in turn, increases the tissue damage even more, which further enhances the release of DAMPs. This vicious cycle is at the base of a cluster of noncommunicable diseases called *inflammatory diseases*.

1.3.2. Inflammatory diseases: Challenges in studying and treating noncommunicable diseases

The majority of inflammatory diseases observed in the general population are immune-mediated inflammatory diseases (IMIDs). Common chronic diseases such as type 1 diabetes (T1D), rheumatoid arthritis (RA), multiple sclerosis (MS), psoriasis and many others, are all characterized by a local or systemic dysfunction due to dysregulation of the normal immune response, which usually originates from genetic malfunctioning.
Thanks to Genome-wide association studies (GWAS), it has been possible to observe an overlap in genetic loci involved in IMIDs. These loci are known as single nucleotide polymorphisms (SNPs) and overlap in 186 regions of the genome between one or more IMIDs. However, the mapping of such loci has been done following statistical models. Consequently, it is not yet possible to prove that a true overlap actually occurs. Yet, it is known that many IMIDs co-occur in the same individuals and families, suggesting that the shared genetic aetiology of these diseases has more to it than mere mathematical coincidence (David et al., 2018).

Genetic malfunctioning might not only be due to IMIDs linked to particular SNPs, but also to epigenetic events. Non-coding RNAs, CpG DNA methylation and histone modifications, are all the epigenetic modification events. These same events have been characterized and reported to be involved in the pathophysiology of autoimmune and inflammatory conditions. Both SNPs and epigenetic events are interconnected because both raise the individual susceptibility for developing IMIDs (Surace and Hedrich, 2019).

Post-inflammation tissue modifications and current anti-inflammatory solutions

Recently, an interesting relation between periodontal inflammation and systemic innate immune response has been demonstrated (Fine et al., 2020). It was suggested that the relation could be due to a tissue remodelling event that happens after the resolution of inflammation. This event has been observed in multiple recent studies. In these studies, it has been shown that even after the resolution of inflammation, there are lingering effects on the tissue affected by it. This phenomenon was thus named "*post-resolution immune system remodelling*" and was first described by da Fonseca et al. (Fonseca et al., 2015; Feehan and Gilroy, 2019). Post-resolution immune system remodelling is characterized by modifications of the original physiological and biochemical state of the affected tissues. The altered state of the affected tissue leaves it more predisposed to developing chronic inflammation, a fact that indicates that tissue immune alterations have taken place (Fonseca et al., 2015).

Overall, these studies suggest that inflammation plays a bigger role than first thought on the development and progression of chronic inflammatory diseases. Currently, there are already available different drugs that are able to dampen symptoms of IMIDs by targeting and inhibiting either certain signal transduction pathways or receptors that can produce proinflammatory cytokines and other inflammatory chemoreactants. Although these approaches have been traditionally used to treat inflammatory diseases and can be very effective at reducing inflammation in the short term, they have been shown to not suffice and to have adverse effects in the long term (e.g. increase in susceptibility to infection due to their dimming effect of inflammatory responses). Therefore, it should become a priority to develop novel preventive and treatment oriented strategies to solve the causes of chronic inflammation. In order to do so there is a strong need to better understand the inflammatory process in inflammatory diseases.

1.3.3. CD36 and its role in atherosclerosis

Although atherosclerosis is a multifactorial disease, its main characteristic is the chronic inflammation of the entire vascular tree. The development of the disease is often due to the coexisting of other pathologies which expose the cardiovascular system to noxious stimuli. Diabetes, hypertension, hypercholesterolemia, hyperhomocysteinemia, or other inflammatory conditions such as aging, rheumatoid arthritis, systemic lupus erythematosus, and human immunodeficiency virus (HIV) can all contribute to the slow onset of atherosclerosis (Tabas et al., 2015). Therefore, atherosclerosis can be considered as an "unresolved vascular inflammatory response" (Galkina and Ley, 2009).

The disease presents itself as a build up of atherosclerotic plaques. Monocytes are the main players in the formation of such plaques. Once monocytes are extravasated into the subendothelial matrix, they are internalized into the vascular tissue; therein, they undergo differentiation into macrophages. The differentiation process allows for the overexpression of a family of the scavenger receptor cluster of differentiation 36 (CD36) (Chistiakov et al., 2016). CD36 is a membrane glycoprotein whose expression is restricted to different types of cells such as monocytes, adipocytes and platelets (Armesilla and Vega, 1994). This scavenger receptor has been shown to play an important role in atherosclerosis (Chistiakov et al., 2016). This particular glycoprotein belongs to the pattern recognition receptor family (PRRs) and has been shown to be involved in several biological processes. It has been shown that CD36 found in macrophages is involved in formation of arterial lesions due to its interaction with oxidized low-density lipoprotein (oxLDL). A high level low-density lipoprotein (LDLs) in the blood leads to their accumulation in the arterial intima (Bentzon et al., 2014). The build-up alerts the blood-circulating monocytes, which start to transmigrate in this site; hereby the monocytes get stimulated to differentiate into macrophages. The resulting macrophages start to upregulate the expression of CD36, with which they internalize oxLDL. The specific oxidized lipids found in the uptaken oxLDL function at this point as ligands of the hormone receptor PPAR-ɣ which in turn further upregulate the expression of CD36, triggering a loop of uptake and upregulation by the macrophages involved (Park, 2014). This particular loop not only leads to the formation of the so called "foam cells", but also to the attainment by some macrophages of a proinflammatory M1-like phenotype (sometime also M2-like phenotype) which is able to secrete high amounts of proinflammatory cytokines such as interleukin-1beta and tumor necrosis factor-α (Bentzon et al., 2014). This lipoprotein-driven inflammatory process, if not resolved, can lead to vascular lesions.

Biomedical engineering DNA and RNA-editing technologies could be used to better understand the inflammatory process in noncommunicable diseases

Biomedical engineering DNA and RNA-editing technologies, may give us a better understanding of not only the innate immune response processes, and of the role of the genes presenting pre-existing unwanted modifications (e.g. SNPs or epigenetic modifications) play in them, but also of the post-resolution immune system remodelling that takes place due to injury or infection.

Using biomedical engineering DNA and RNA-editing technologies would make it possible to study these different aspects of some noncommunicable diseases by artificially disrupting the genes or proteins of interest that are involved in the different underlying mechanisms. This would allow us to observe the unfolding of events induced by the disruption in a controlled environment.

An example of such disruption could be the knockout of CD36 to study atherosclerosis.

2. RESEARCH AIMS

Given the access we have to different biomedical engineering editing tools, it is of utmost importance to collect together and make sense of what we already know about them, before proceeding in a biomedical research project that involves any of these tools. Moreover, since gene-editing is a promising new technology in medicine (e.g. gene-therapy), it is worth investing some time in examining a bit closer what potential the aforementioned tools hold in translational medicine for noncommunicable diseases. However, at the moment, the information available is rather scattered and numerous, and the amount of work to collect and make sense of it all is painstaking and time consuming.

Therefore, this project was created to facilitate the daunting task of introducing oneself in the vast body of knowledge of CRISPR/Cas9, prime Editing, and programmed RNA-editing technologies.

The present thesis can thus be seen as a theoretical microscope that focuses at different magnification levels on the vast topic that is biomedical engineering gene-editing technologies. At the lowest magnification level, the thesis aims to first provide a comprehensive account of the most current gene-editing and RNA-editing tools. A second aim is then to focus further into providing insights into the main opportunities and challenges of applying these tools in translational medicine. Lastly, at the highest magnification level, the thesis aims to explore the possible future applications of gene and RNA-editing tools in the most common noncommunicable diseases (e.g. studying atherosclerosis by targeting the scavenger receptor CD36 in macrophage-like cell lines). For the latter, there will be provided an example model. The emphasis of the model will be on how to design good RNA guides for CRISPR/Cas9 using different bioinformatics methods.

3. METHODOLOGY

3.1. Methods used for the biomedical narrative review of three novel biomedical engineering editing tools for their application in translational medicine

In the biomedical narrative review part of this thesis, the focus is to find out the potential in translational medicine use of three different novel genetic engineering tools, namely: CRISPR/Cas9, prime editing, and RNA-editing. In particular, the potential will be determined by the overall target editing capability when it comes to *in vivo* biomedical research, and preclinical trials if available. Therefore, the only studies taken into consideration are those where the experiments are performed *in vivo,* in human primary cells or organoids, and in pre-clinical trials.

The research question we want to try to answer with this review is: "What opportunities and challenges do CRISPR/Cas9, prime editing and programmed RNA-editing present and what potential application do they have in translational medicine?"

The studies taken into consideration for this review were selected based on how recently they were published (from 2015 or later), relevance of content (only biomedical studies involving gene- and RNA-editing; *in vivo* studies; *in vitro* studies but only if they involved the use of organoids, or human primary cells studies; and pre-clinical trials when available). The journals were selected based on their H-index (not lower than 100). The literature research was performed on PubMed and Google Scholar, searching for the following keywords: CRISPR/Cas9, Prime Editing, programmed RNA-editing, CRISPR/Cas9 *in vivo*, Prime Editing *in vivo,* programmed RNA-editing *in vivo*, CRISPR/Cas9 disease models, Prime Editing disease models, programmed RNA-editing disease models.

3.2. Designing guide RNAs to knockout CD36 in monocytes using CRISPR/Cas9

RNA guides (gRNAs) are the main protagonists in the gene-editing process. The model presented in this thesis aims to be an example of how to design good gRNAs for CRISPR/Cas9. Without good gRNAs, it is hopeless to successfully identify any given gene target, even less be able to properly knockout its expression. Therefore, the model will start by providing a detailed account of the design process for CD36 gRNAs.

3.2.1. Designing RNA guides for CRISPR/Cas9

Selecting the most effective sequences for guide RNA using different bioinformatics tools

The aim of this *in silico* experiment was the identification of the most effective single guide RNA (sgRNA) sequence that can be used for guiding the CRISPR/Cas9 knockout of CD36. Three score systems were used to this end:

1. The Vienna Bioactivity CRISPR score (VBC): www.vbc-score.org
2. The Broad Institute Doench's scoring model CRISPRko (GPP): https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design
3. CHOPCHOP: https://chopchop.cbu.uib.no/

First, the sequences were selected based on their presence in all three systems (other sequences present in only two systems or in just one system were discarded) and then ranked based on their order in the respective systems. Second, the sequences were ranked based on each system's most important parameters for effective CRISPR/Cas9 experiment.

1. *The Vienna Bioactivity CRISPR score (VBC)*

On the VBC score system (The Vienna Bioactivity CRISPR score) the following was taken into consideration for the ranking: (1) *The sgRNA activity score*; (2) *Frameshift ratio inDelphi*; and (3) the *Bioscore*.

(1) *The sgRNA activity score* refers to the single-guide RNA activity on-target; the score for this parameter is set to be between 0 = bad (low activity) and 1 = good (high activity) (Michlits et al., 2020).

(2) *The frameshift ratio inDelphi* parameter is a computational prediction module that allows *in silico* studies to anticipate the template-free CRISPR/Cas9

editing outcomes, and it is based on logistic regression or deep learning (Naert et al., 2020). This score is important because frameshift indels generated by CRISPR/Cas9 lead to the wanted knockout outcome of loss-of-function of the targeted gene (Chen et al., 2018); thus, the higher the score the higher the probability to succeed in the knockout of the target.

(3) The last score taken into consideration in the VBC score system, was the *Bioscore*, which is the combination of different features (Pfam domains, DNA and a.a. Conservation, a.a. Identity and gene structure) in one single score. These features predict the phenotypic effect of a generated indel (Michlits et al., 2020); the score for this parameter is set between 0 = bad (low effect) and 1 = good (high effect).

2. *The Broad Institute Doench's scoring model CRISPRko (GPP)*

On the GPP CRISPRko system (The Broad Institute Doench's scoring model, now re-named CRISPick) (Hanna and Doench, 2020) it was taken into consideration the target cut score (in percentage), off-target rank and on-target rank set by the system for each sequence and the on-target efficacy score. The target cut score is the percentage of the target gene that comes before the cut (5'->3'). Thus, the higher the percentage the more is cut out of the target, which results in higher probability of effective knockout. The off-target and on-target ranks are generated based on the so-called "On-target Rule Set" which is a model based on the "Rule set 2" developed by Doench, Fusi et al. (Doench, Fusi et al., 2016). This model makes it possible to calculate the "on-target" efficiency of a given sgRNA, represented by the efficacy score in this system. Thus, the higher the sequence is ranked in the on-target and off-target parameters, the more specific is that particular sgRNA. Moreover, the higher the on-target efficacy score, the higher is the probability that the knockout would be effective on the target gene.

3. *The CHOPCHOP score system*

The CHOPCHOP score system takes into consideration the GC (guanine and cytosine DNA bases) content (in percentage), the self-complementarity score, the MM2 and MM3 scores, and the efficiency score. The GC content is the score that indicates the effectiveness of a given sgRNA based on its GC percent. It was shown that the most effective sgRNAs have a GC content between 40 and 70% (Wang et al., 2014; Tsai et al., 2015). Thus, the higher the percentage of GC the more effective the sgRNA taken into consideration will be. The self-complementary is the parameter that indicates the guide RNA efficiency based on its self-complementarity between its own sequence and the RNA backbone, which, if present, can inhibit gRNA efficiency (Thyme et al., 2016). The number given in the system results for this parameter indicates how many regions of self-complementarity are predicted for that particular sgRNA. The self-complementarity should be 0. The MM2 and MM3 represent how many off-targets each target site has with 2 and 3 mismatches. The off-target transcripts with the different mismatches' numbers are those that a given gRNA may bind to, outside of the target gene.

There is no universal rule for predicting CRISPR/Cas9 sgRNA off-target effects. However, several studies have shown that the sgRNA guided-cut proximity to the protospacer adjacent motif (PAM) can disrupt the editing of the target sequence. The PAM is a short sequence found as part of a DNA sequence and has an important role on the cleavage efficacy of Cas9. PAM is important for target recognition by Cas9. Without it the protein is not able to identify the target sequence. Therefore, the more and the closer the mismatches are to the PAM sequence, the less effective the knockout will be (Hsu et al., 2013).

A study by Cong et al. (2013) indicates that single-base mismatches up to 11 bp away from the PAM (on the 5' strand) completely inhibits Cas9 cleavage action on target genes. This is why CHOPCHOP searches for mismatches generated by a given sgRNA across all bases upstream of the PAM sequence in the target gene (Montague et al., 2014). Therefore, the lower the number of mismatches, and the further they are, the higher the sgRNA will be ranked. The efficiency score in CHOPCHOP is the same as the "on-target efficacy score" in the GPP CRISPRko system due to the fact that this score is based on the Doench metric method.

An overall ranking was made based on the above different ranking systems, and the three top sequences were picked (see Appendix 1., 2., and 3.).

Making gRNAs compatible with the lentivirus vector (LentiCRISPR)

The aim of this experiment was to design gRNA oligonucleotides (short single strands of synthetic DNA) for the designed gRNAs in order to make them compatible with the lentivirus vector *LentiCRISPR.* This vector is able to infect different types of mammalian cells.

For making gRNAs compatible with the lentivirus vector, the protocol for cloning into the lentiviral transfer plasmid from ZhangLab (ZhangLab; Sanjana et al., 2014; Shalem et al., 2014) was followed.

The gRNAs oligos have to be synthesized together with Cas9 in order for the lentiCRISPR system to co-expressing a single guide RNA (sgRNA) and a mammalian codon-optimized Cas9 nuclease in the infected cells. The sgRNA facilitates genome editing by guiding the Cas9 nuclease to the right target sequence in the genome (Shalem et al., 2014).

A pair of annealed gRNA oligos (i.e. target fragment) can be cloned into the sgRNA scaffold in the LentiCRISPR (**Fig. 1**) and can be digested and synthesized together with Cas9 using a restriction enzyme (ZhangLab; Sanjana et al., 2014; Shalem et al., 2014).

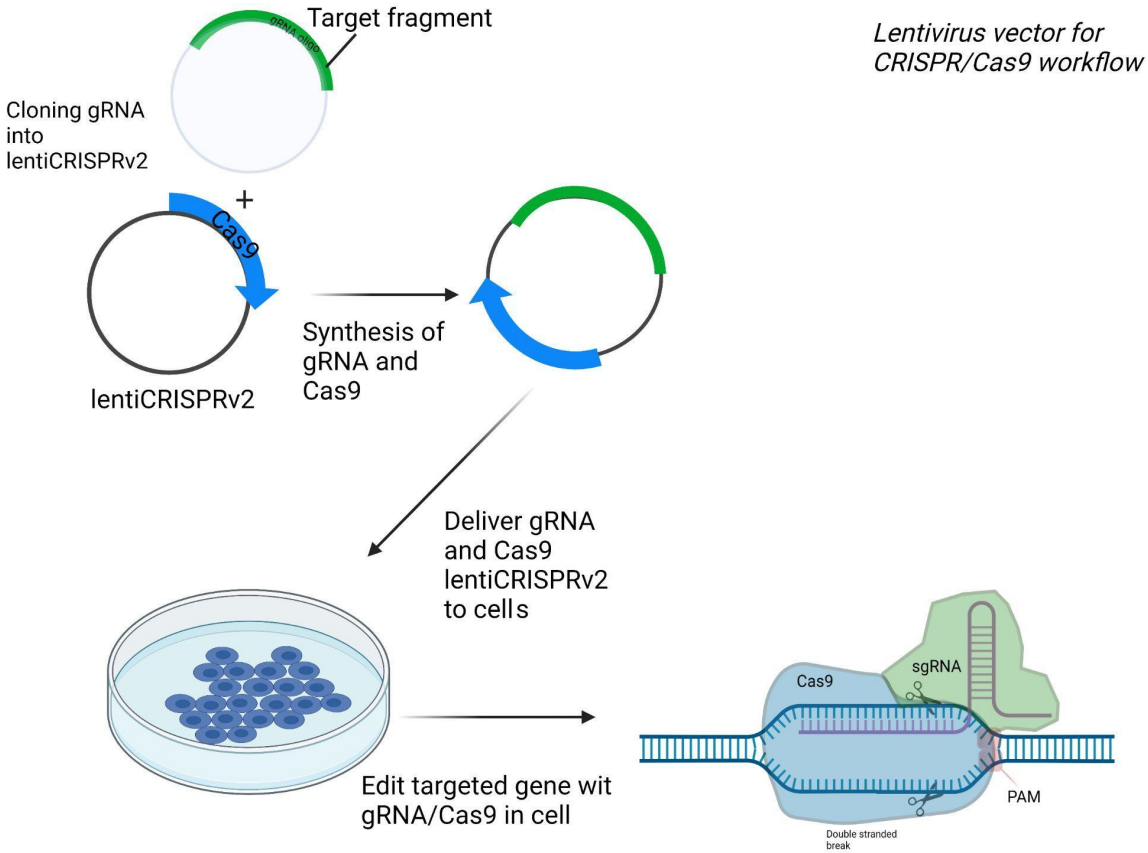

Figure 1. Illustration of the general workflow for synthesis of the guide RNA together with the Cas9 enzyme in the same lentiCRISPRv2 plasmid. The target fragment (gRNA) is cloned into the plasmid where the Cas9 sequence is already present. The two sequences are then synthesized *in vitro* into the same plasmid. The plasmids will then be replicated by culturing the lentivirus which will then be exposed to the cells to infect them in order to transfer the plasmid. Adapted from: "How to synthesize your gRNAs for CRISPR" by Pellegrini, 2016, Benchtalk.
(source: benchling.com/2016/02/23/how-to-synthesize-your-grnas-for-crispr/ accessed on the 13th of May 2021) © Copyright 2021 Benchling. Created with BioRender.com

Since the target sequences selected in the sgRNA design process must be cloned into the LentiCRISPRv2 two oligos must be designed using the designed gRNA sequences without including the PAM sequence.

Methods for designing primers to amplify the gRNAs sequences

In order to detect the presence of gRNA oligos in the lentiCRISPRv2 vector once the gRNAs are cloned in the plasmid, it is necessary to design primers that are specific for the target sequence. With these primers it is possible to amplify the target sequence by polymerase chain reaction (PCR). The primers can then help to modify the target sequence by either deletion or insertions. The target sequence of gRNA can be easily introduced through the forward primers, which also introduce the T7 promoter into the template.

The aim of this experiment was to design primers for amplification of the designed gRNAs sequences and to design the oligos compatible with the lentivirus DNA for cloning of gRNAs into the plasmid.

An adapted version of the CRISPR fly design protocol ("CRISPR fly design, gRNA IVT: Production of gRNAs by in-vitro transcription (IVT") was followed to design DNA primers for our vector.

To design the primers, the CD36 complete gene sequence was gathered by visualizing it on the UCSC Genome Browser (UCSC "Genome Browser Home") from the link provided on the CHOPCHOP target site search result page for CD36.
By clicking on the very first sequence provided on the UCSC page, the user gets redirected to another page. In this page the following parameters were selected: 5' UTR Exons, CDS Exons, 3' UTR Exons, Introns, Downstream by 1000 bases, One FASTA record per gene,

Exons in upper case, everything else in lower case. This process leads to a long FASTA sequence which includes both introns and exons of CD36 gene sequence. In order to design the primers for each target sequence, only the exons associated with those sequences were selected (in this case, exon 10 and exon 14), copied and pasted on the bioinformatic tool provided by NCBI for finding specific primers (Primer-BLAST). The selection did not include only the exon sequence but also some of the intron sequence upstream. It is important to include some of the intron part in order to avoid designing the primer too close to the exon. If this step is overlooked, there might not be an optimal coverage of the entire exon sequence during the amplification process, which ultimately could lead to inadequate transcription. Once the sequence was selected and copied on the Primer-BLAST, the program was run. Several primers were gathered and the two top ones were picked to be checked on the in-silico PCR program found on UCSC. This was done for each target sequence.

In order to design these primers to each target sequence it was added the T7 promoter sequence TAATACGACTCACTATAG - to the beginning of each target sequence, and the gRNA core - GTTTTAGAGCTAGAAATAG at the end of each sequence. The six extra nucleotides added before the T7 promoter are random and are only included to increase the "landing" site for T7 RNA polymerase.

The final oligos were designed following the - 5' - 3' direction of replication. The oligos were designed based on the target site sequence (20nt) by adding at the beginning of each sequence (5' end) the sequence: CACCGN (in green); in the complementary sequence it was added an extra Cytosine (3' end) and the sequence CAAA (in blue) (5' end) (**Table 1**).

Table 1 Oligos design process illustrated

Oligo 1 →	5' - CACCGGATTCCTTTCAGATTAACGT - 3'	
	3' - CCTAAGGAAAGTCTAATTGCTCAAA - 5'	**← Oligo 2**
Oligo 1 →	5' - CACCGGAATCCGACGTTAATCTGAA - 3'	
	3' - CCTTAGGCTGCAATTAGACTTCAAA - 5'	**← Oligo 2**

Oligo 1 → 5' - CACCGTCATTTCTATCAGGCCAAGG - 3'

3' - CAGTAAAGATAGTCCGGTTCCCAAA - 5' ← Oligo 2

3.2.2. Selecting method for testing CD36 gRNA in cells

Since macrophages are the main cells involved in atherosclerosis and expressed CD36, there was a need to find the most suitable macrophage-like cell line for testing CD36 gRNAs. In particular, there was a need to use a cell line type that expressed detectable CD36, and that could be easily assayed for CD36 expression. Therefore, it was decided to compare the cell line U937 with the commonly used cell line THP-1. U937 cells are supposed to present a higher CD36 surface expression than THP-1 cells in undifferentiated cells (Alessio et al., 1996).

THP-1 cells

THP-1 cells allow the investigation of monocyte structure and function (Bosshart and Heinzelmann, 2016). The cells used here were derived from the peripheral blood of a 1 year old male with acute monocytic leukaemia.

U937 cells

U937 cells are an in *vitro* model similar to THP-1 cells. The cells used here were derived from malignant cells of a pleural effusion of a 37 year old caucasian male with diffuse histiocytic lymphoma.

3.2.3. FACS analysis for assessing CD36 gene expression in both THP-1 and U937 cells

Principles of FACS analysis

Fluorescence-activated cell sorting (FACS) analysis is a type of flow cytometry which is a laboratory method that allows for measuring the optical fluorescence of a single cell. The different components of the cells are made fluorescent thanks to antibody staining. The antibodies are specific for the different cell components. Cells are usually stained with fluorescence probes (called fluorochromes) conjugated with an antibody, which are able to stain, and thus make detectable by a FACS machine, cell components usually not visible. A light source is then used to analyse the light scattering and fluorescence emission derived from either antibodies (or dyes), which are able to give data on size, granularity and the fluorescence features of cells.

Antibody titration is needed to perform a proper FACS analysis. This process helps to determine the concentration of antibodies needed for separation of cell population in a given sample based on expression levels of a given protein. Therefore, it is crucial to use the right concentration of a given antibody for obtaining robust results from the flow cytometry analysis. A concentration too low could result in a poorly stained marker of interest, and can also allow too much clumping of positive cells and negative cells. On the contrary, too much antibody is not good either, since it can increase non-specific binding, which then increases the spread and the background noise of the negative population (UWCCC Flow Cytometry Laboratory, “Titrating Antibodies for Flow Cytometry”).

In the following experiment it was used FITC which are a type of fluorochromes used for labeling antibodies, due to their easy reactability and moderately stable conjugates with proteins in cells. This particular property of FITC makes it crucial to add the dye to an aliquot of DPBS and incubate before adding it to the washed cells, since the dye would bind to any proteins in solution.
Fluorochromes present a wide emission spectrum which results in overlap of signals. In order to avoid this event, the overlap is corrected by subtracting unneeded emission signals, thus compensating the overall emission. Therefore, it is important to use compensation beads for fluorescence compensation. This prevents interference from fluorochrome channels that were not assigned to be specifically measured.

Assessing expression of surface CD36 in undifferentiated U937 cells and differentiated U937 and THP-1 cells by flow cytometry

Two different experiments were performed to assess the CD36 expression level in two different types of macrophage cell lines. The second experiment was performed by the thesis supervisors Nadra J. Nielsen and Erlend B. Tande.

Assessing expression of surface CD36 in undifferentiated U937 cells

The aim of this experiment was to assess expression of surface CD36 in undifferentiated U937 cells. This first experiment was meant to select the right concentrationof antibody needed for expression analysis and to calibrate the FACS machine for the later analysis of CD36 on both cell types.

Cell culture: THP-1 cells

The cells were cultured in RPMI Medium 1640 (1x) which had been mixed with 10% FCS, 1% Penicillin Streptomycin (P/S) (antibiotic used to make sure that the medium is completely sterile), plus extra glutamine beta-Me 0.05 μM (to protect cells by reducing oxidation levels in cells). An aliquot of subcultured THP-1 cells was kept in an incubator at 37 degrees Celsius and at a 5% CO2 concentration. The CO2 concentration is kept at this level to make sure that the pH of the cell culture keeps stable at around 7-7.4 pH, which is the standard pH of human blood. Every 18 hours the cells divide and continue to do so exponentially. Therefore, the subculture of cells was split regularly in order to avoid overcrowding the cell culture, which, if failed to be done, would lead to the death of the entire culture after some time. The concentration of cells in a 25 ml medium suspension were kept between 200.000 and 800.000, which is the limit for THP-1 cell survival.

Cell culture: U937 cells

The cells were cultured in RPMI Medium 1640 (1x) which had been mixed with 10% FCS, 1% Penicillin Streptomycin (P/S), and glutamine 2 μM, plus 10% Foetal Bovine Serum (FBS). An aliquot of subcultured U937 cells was kept in an incubator at 37 degrees Celsius and at a 5% CO2 concentration. Every 72 hours the cells divide and continue to do so exponentially. Therefore, the subculture of cells was split regularly in order to avoid overcrowding the cell culture. The concentration of cells in a 25 ml medium suspension were maintained between 800.000 and 1 million, which is the limit for U937 cell survival.

Staining undifferentiated U937 cells for fluorescence assisted cell sorting (FACS)

The U937 cells were split in two different samples in order to get 1 million cells/sample. The first sample was used as viability staining control for easily excluding dead cells from the flow cytometry analysis. This is an important step as dead cells can non-specifically bind to antibodies, which can compromise the final results. The second sample was instead used for the titration process.

Preparation of the dead cell sample

The aim of this experiment was to kill cells to then combine them with live cells in order to get positive and negative control for compensating the viability staining. In the FACS analysis there needs to be a positive peak (provided by the dead cells) and a negative peak (provided by the live cells) in order to be able to identify dead cells and exclude them. The viability dye used for the staining was Fixable Viability Dye eFluor™ 780 (cat: 65-0865-14, Invitrogen) which allows to easily exclude dead cells when extracellular targets are being studied.

The first sample was spun at 1500rpm for 5 minutes at 4 degrees Celsius. The supernatant was removed and the cells pellet was washed with Dulbecco's phosphate-buffered saline (DPBS) and then spun again with the same centrifuge conditions. The cell pellet was resuspended in a solution of DPBS and of 96% ethanol, this was done to kill as many cells as possible. The solution was then incubated on ice for 5 minutes. After incubation, a sample was taken from the solution and used for cell counting and viability analysis on Invitrogen™ Countess™ Automated Cell Counter. The solution was spun twice and the pellet was washed each time with a small amount of DPBS. The cells were then resuspended in a dilution of viability dye and DPBS. The cell-dye dilution was then incubated for 10 minutes on ice before being analysed in the FACS machine. Compensation Beads (cat: 01-1111-42, Invitrogen) were stained in parallel for compensation.

Preparation of the live cells sample: Antibody titration

The second sample was spun (same centrifuge conditions as the first sample) and the pellet was washed once with DPBS. The cells were then resuspended in a solution of FACS buffer (2% BSA/2%A+v.i./DPBS) and FC block mix. The solution was incubated for 10 minutes at roughly 4 degrees Celsius (the tube was kept on ice).

The U937 cells (2 million cells/sample) were treated with a FACS buffer (2%BSA/2% heat-inactivated A+ serum./PBS) and then incubated in FC block (Fc Receptor Binding Inhibitor Polyclonal Antibody; cat: 14916173, Invitrogen) (5ul/sample) for 10 min at 4C.

The cells were stained with different volumes of FITC anti-human CD36 Antibody (FITC anti-CD36 Ab; cat: 336204) or FITC Mouse IgG2a, κ Isotype (FC) Antibody (FITC Control Ab; cat: 400210) (i.e.: 5 µl, 2.5 µl, 2 µl, 1.5 µl, 0 µl).
After incubation for 10 min, the samples were washed twice with DPBS and subsequently stained with viability dye efluor 780 (APC-Cy7)(cat: 65-0865-14, Invitrogen) and then incubated on ice for 10 minutes. The cells were then washed once with the FACS buffer before cell staining was assessed on FACS BD LSR II machine.

FACS analysis: Machine calibration and gating strategy
The FACS machine was calibrated using the compensation beads sample (FSCvs SSC) and the live/dead cells stained with viability dye. Viable cells were "gated" (10000 cells were counted to be in the gate).

Assessing expression of surface CD36 differentiated U937 and THP-1 cells by flow cytometry

The preliminary aim of this experiment was to assess the expression of surface CD36 in PMA-differentiated U937 and THP-1 cells. This experiment was meant to test the findings of Alessio et al. (Alessio et al., 1996), which reported that U937 cells present a higher level of CD36 expression than THP-1 cells. The final aim of this experiment was to choose the cell line with higher levels of CD36 expression. The cells selected would then be deemed fit to be used as a model to knockout CD36 with CRISPR/Cas9.

Differentiation of both cell lines and analysis by flow cytometry
Both THP-1 cells and U937 cells were differentiated by exposing them to PMA for 3 days. The differentiated cells were then analysed for viability and then split into two samples (~0.25mill cells/sample for THP-1 cells; ~0.5mill cells/samples for U937 cells).
The cells were stained and analysed on FACS BD LSR II following the same procedure as presented for the aforementioned experiment.

4. RESULTS

4.1. A comprehensive account of the most current gene- and RNA-editing tools

Results for the biomedical narrative review

The following section provides a comprehensive account of the most current gene-editing and RNA-editing tools.
The studies reviewed have been divided in categories and reported in **Table 2.** The studies have been categorized based on the type of biomedical engineering tool used, the cell delivery method used, and the cell type and disease studied. In addition, in **Table 2.** each study's own results are summarized.

The delivery method used for transfecting cells is crucial for the editing precision and safety
The main challenge that these novel editing tools share is the delivery to the cells. Consequently, in the last years there has been an increased attention towards the use of more transient, efficient and scalable delivery systems, which can better prevent off-target events and immune reactions.
A fine example are lipid nanoparticles (LNPs) which are used as alternative cell delivery systems to the traditional ones, e.g. viral vectors and electroporation. Viral vectors are vectors that rely upon the use of viruses such lentiviruses and adenoviruses that can infect cells and integrate engineered plasmids in them; whilst electroporation is a type of "physical" delivery system used to transiently disrupt cell membrane lipid bilayers via an electric field, in order to enhance permeability. Although this method is quite efficient for delivery of CRISPR/Cas9 to cells, it causes a high rate of cell damage and cell death (Song et al., 2021). These traditional delivery systems are not optimal given the fact that viral vectors are immunogenic (Sakurai et al., 2007; Milone & O' Doherty, 2018) and that electroporation can result easily in cell damage and death.

The key attributes of LNPs (e.g. non-immunogenic, precise delivery, cell-tolerant) make them an optimal delivery system because they meet all the criteria necessary for safe and efficient gene-editing. Before CRISPR/Cas9, these particles had already been validated in both pre-clinical and clinical trials for the delivery of small interfering RNA (siRNA) (Thi et al., 2014; ClinicalTrials.gov ID: NCT01960348; Finn et al., 2018) and mRNA (Richner et al., 2017, Magini et al., 2016; Finn et al., 2018). Although promising, most of the nanoparticles

designed so far have been shown to be rather inefficient when it comes to clinical uses (Finn et al., 2018).

Two major recent studies see the successful use of these types of nanoparticles for delivery of CRISPR/Cas9. The studies were presented by Finn et al. (2018) and Xu et al. (2018). In both studies the LNPs used were specifically designed based on specific experiment parameters, and resulted in a robust and persistent in vivo genome editing in mice (for further insights, see subchapters 4.3.1 and 5.2.1). Both LNP systems deliver a Cas9 mRNA (mCas9) instead of the standard single Cas9 protein. The mCas9 used in these studies is designed to lower cellular innate immunity response compared to the standard single Cas9 protein. Despite this, it can still trigger an immunogenic response, above all if the delivery is systematic, as it usually is in a clinical setting.

New formulation of LNPs have made them less immunogenic

In order to prevent any adverse immune reactions, in the last few years there has been a growing interest in formulating stealth PEGylated liposomes, using a process called PEGylation. This process involves biochemical modifications of bioactive molecules with poly-ethylene glycol (PEG). PEG is a non-toxic and non-immunogenic polyether diol layer (Gupta et al., 2019).

A recent study by Jubair et al. (2021) has shown a promising use of these liposomes for treatment of human papillomavirus (HPV) without causing post-editing immunity . However, the post-editing protection has been shown only on immunocompromised mice (Jubair et al., 2021). Jubair et al. had previously presented in 2012 the formulation of Cas9/gRNA plasmids coated in PEGylated liposomes via the hydration of freeze-dried matrix (HFDM), which resulted in protection of the Cas9-cargo, allowing it to evade the detection by the immune system. The use of HFDM PEGylated liposomes is promising for CRISPR therapeutics, thanks to its efficient delivery to target organs, superior stability, reduced renal clearance and prolonged circulation time (Milla et al., 2012; Jubair et al., 2021), which results in longer sustained editing.

Another similar example of use of liposomes as delivery method for CRISPR/Cas9 is the novel core-shell nanostructure, liposome-templated hydrogel nanoparticles (LHNPs) developed by Chen et al. back in 2017 (Chen et al., 2017); this method has been shown to deliver the Cas9 protein effectively in cultured cells. The LHNPs core is formed by polyethylenimine (PEI) hydrogel which is a similar biomaterial to the PEG used in the HFDM liposomes study.

4.1.1. CRISPR/Cas9: The "search-and-cut" editing tool

How it works

Currently the system can be designed by using "humanized" versions of *Streptococcus pyogenes* or *Streptococcus thermophilus* Cas9 proteins, which are usually coexpressed with custom-designed single guide RNAs (sgRNAs). These RNA guides vary because they are differently designed based specifically on the target of interest. The Cas9-sgRNA complex is then transfected to cultured cells by different means (e.g. lentiviral transfection method or electroporation). The complex is then able to target a specific gene sequence thanks to the sgRNA. The complex can then"cut" the target sequence out using the Cas9 enzyme. The resulting alterations on the edited DNA sequence takes place due to the stimulation of gene-editing by the nonhomologous end joining repair (NHEJ) process or gene replacement by homology directed repair (HDR) process caused by site-specific double-strand DNA break (DNBs) (**Fig. 2**).

Depending on the target, this complex editing efficacy can reach up to 80% or more in vitro (Doudna and Charpentier, 2014), but only if multiple parameters are optimal (e.g. sgRNA sequence design, duration of nuclease exposure, DSB repair mechanism, etc.). The main challenges of this genetic engineering method lie mostly on its off-target effects, its inconsistent editing efficacy, and its immunogenicity (Rosenblum, 2020).

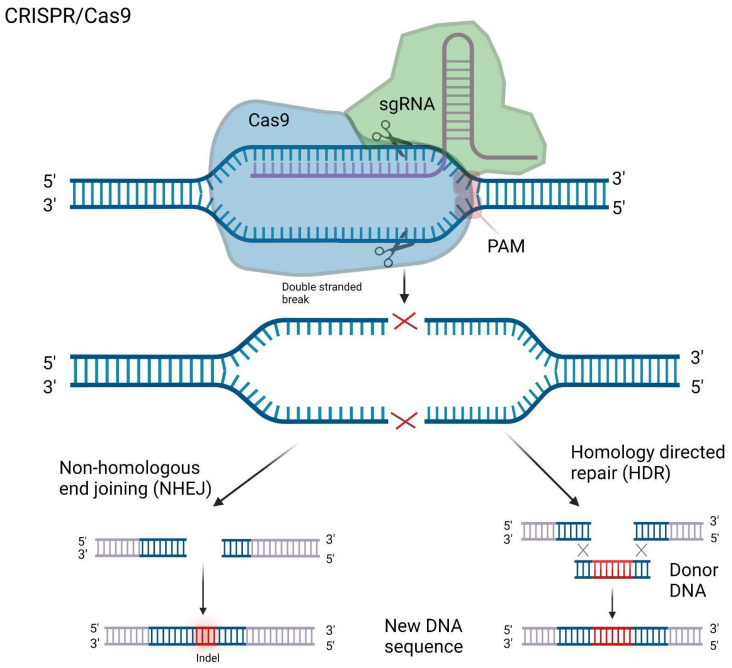

Figure 1. Double-stranded break performed on DNA by CRISPR/Cas9-sgRNA complex with the two possible outcomes. The CRISPR/Cas9 mediated gene-editing is possible thanks to the recognition by the single guide RNA (sgRNA) of the target sequence. It is able to do so by individualizing the PAM sequence specific for the target sequence. Once the target is identified, the Cas9 DNA endonuclease is activated which performs a

double-stranded break. The break can trigger two different DNA damage repair processes: The non-homologous end joining repair (NHEJ), or the homology directed repair (HDR) if a donor DNA is available. The NHEJ is an error prone pathway which can result in indels in the new DNA sequence. Adapted from "Science and Bioethics of CRISPR/Cas9 Gene Editing: An Analysis Towards Separating Facts and Fiction" by Cribbs and Perera, 2017, *Yale Journal of Biology and Medicine,* 90: 625-634. Copyright © 2017 by University of Oxford. Created with BioRender.com

Current applications

In the first study reported in **Table 2.**, Finn et al. used a biodegradable lipid to form LNPs co-formulated with both Spy Cas9 mRNA and a chemically modified sgRNA, which further boosted the levels of activity of the Cas9 protein once delivered in a single dose (up to 52-week period). The formulation of this system, called "LNP-INT01", has been demonstrated to be able to target endogenous genetic defects in mouse primary hepatocytes. The system can deliver CRISPR/Cas9 components for targeting of mouse transthyretin (*Ttr)* gene, the homolog of a gene-editing target for treating amyloidosis in humans. The study does not take into consideration off-target effects due to the fact that undesired editing of non-hepatic tissue with modifications aimed to hepatocyte-specific expressed genes should not have any functional consequence. The efficacy of the editing was estimated to be circa 70% with a single injection in rats (Finn et al., 2018).

The second study reported in **Table 2.** presents a designed LNP, an optimized cationic lipid-assisted nanoparticle (CLAN), formulated for specific intestinal macrophages for editing of their *NLRP3* gene. In this study it was shown that the frequency of six possible off-target sites was lower than 0.5%, and indel frequency in the NLRP3 locus was 58.6%. The editing efficacy of knockout was estimated to be 56.8% for a single injection (Xu et al., 2018).

The principles of uptake and editing effects are similar in the two systems, where LNPs are able to interact with serum proteins, which facilitates the uptake by cells of the nanoparticles in a receptor-mediated manner (Akinc et al., 2010; Finn et al., 2018).

The third study reported in **Table 2.** involves the use of CRISPR/Cas9 for treating Transfusion-dependent beta-thalassemia (TDT; CLIMB THAL-111) and sickle cell disease (SCD; CLIMB SCD-121) by targeting the *BCL11A* enhancer which is linked to the transcription factor BCL11A that represses γ-globin expression and fetal hemoglobin in erythroid cells (Frangoul et al., 2021; ClinicalTrials.gov ID: NCT03655678 for CLIMB THAL-111 and NCT03745287 for CLIMB SCD-121). Potential off-target sites were identified before the trial, using sequence similarity (computational) and laboratory-based methods. According to the results gathered using these methods, no evidence of any off-target

editing was reported. The allelic editing frequencies were estimated to be 68.9% for the TDT patient and roughly 80% for the SCD patient (Frangoul et al., 2021).

The fourth study reported in **Table 2.** involves the development of a CRISPR/Cas9 editing method that is being used in a preclinical trial with 18 adults and pediatric participants (Editas trial), suffering from congenital blindness. The preclinical study (ClinicalTrials.gov ID: NCT03872479) for treating congenital blindness is still at its earliest stage. Therefore, it is not yet known if there are any off-target effects nor what real editing efficacy of the treatment is at the genomic level. In this study, reported on *Nature Biotechnology* ("First CRISPR therapy dosed", 2020), the patients are given the first ever *in vivo* CRISPR-based therapy (ClinicalTrials.gov ID: NCT03872479). The study involves a de-novo technique for *in vivo* editing by subretinal injection (in a single eye) of EDIT-101 (also known as AGN-151587) which contains a construct of the adenovirus vector AAV5 with two guide RNAs (gRNAs). The guides are designed to target the IVS26 mutation in the CEP290 gene which causes Leber congenital amaurosis (LCA), a retinal degenerative disease. The gRNAs are combined with DNA encoding the Cas9 enzyme under a promoter specific to particular photoreceptor cells found in the retina.

4.1.2. Prime editing: A CRISPR/Cas9-based "search-and-replace" method

How it works

Prime editing is a CRISPR/Cas9 based method, but it is not just a "search-and-cut" genome editing like CRISPR/Cas9, but rather a "search-and-replace" one. This technology is able to do the search-and-replace by using an RNA-programmable nickase (*S. pyogenes* Cas9-H840A) fused to a reverse transcriptase (e.g. engineered M-MLV RT) and prime editing guide RNA (pegRNA). The pegRNA is important because it specifies both the genomic target and edit sequence, and thus contains a DNA-targeting and a template repair domain. Prime editing follows the same principle of CRISPR/Cas9 where a DNA sequence is identified and nicked. However, in this case the nick happens only in one strand. The nicked target DNA undergoes reverse transcription primed by an editing template carried by a prime editing guide RNA (pegRNA). The editing template contains an RNA edited version of the target gene that contains the error to be corrected. The Cas9 enzyme is fused to the reverse transcriptase enzyme. This allows for the nicking and the reverse transcription to take place at the same time. The pegRNA is able to attach itself thanks to the primer binding site (PBS) present in the editing template (pegRNA). Once anchored, the pegRNA is used as a template for synthesizing corrected DNA by the reverse transcriptase as illustrated in **Fig 2.**

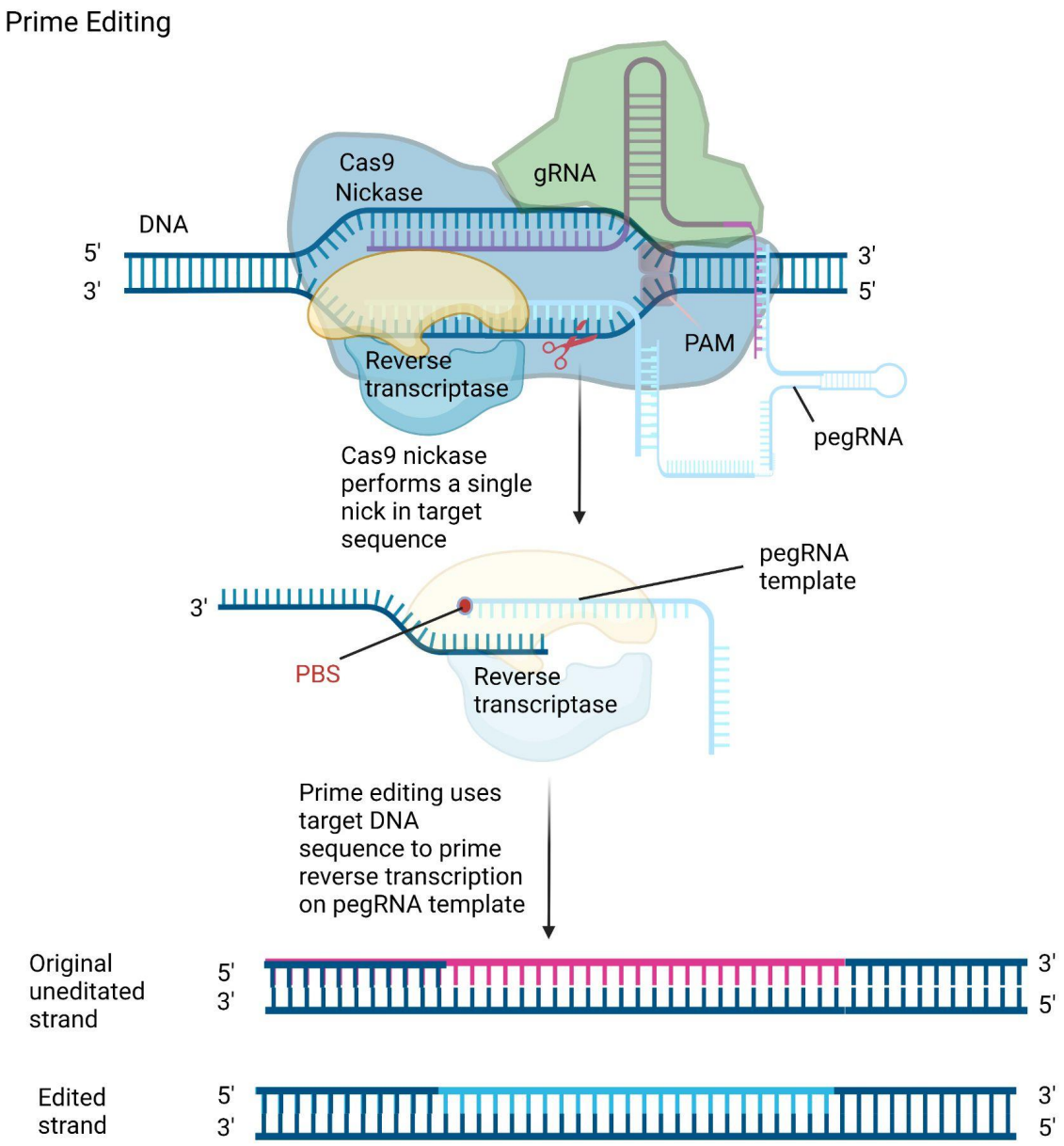

Figure 2. Simplified prime editing technique illustrated. The Cas9 nickase is guided by the gRNA to the target sequence where it is able to perform a single strand nick. Once the target is identified and cut the pegRNA attached to the gRNA is able to provide the template for the reverse transcriptase to replace the nicked target sequence. Adapted from: "Prime Editing: Game Changer for Modifying Plant Genomes" by Marzec and Hensel, 2020, *Trends in Plant Science,* 25(8): 722-724. Copyright © 2020 by Elsevier Ltd. Created with BioRender.com

The biggest advantages that this new base-editing technology provide over the older ones, is its ability to generate more than the usual four transition mutations precise base-editing. It can achieve up to 12 possible base-to-base conversions, and combinations in human cells. Additionally, it has more laxed PAM requirements, thanks to its no "bystander" editing capability (i.e. does not require to identify precise position of PAM for editing). Since this sequence differs greatly between systems, it is difficult to predict. But with PE there is no need to do so (Anzalone et al., 2019). These revolutionary qualities make PE a potentially strong candidate for future treatment of disease-causing mutations found in large genes. Furthermore, it could be successfully applied to autosomal dominant diseases where there is a need to silence or ablate the gene causing the disease. Despite the fact that this gene-editing technology still is in its infancy, its optimization is currently in progress to improve its targeting and efficacy (Kantor et al., 2020). Another advantage reported of this genetic editor resides in its ability to generate precise edits in different types of cells. The main challenges are similar to those presented for CRISPR/Cas9 (e.g. off-target effects, delivery problems etc.) (Yan et al., 2020).
The clinical potential of PE is enormous, and it is estimated that it could correct up to 89% of thousands of pathogenic human single nucleotide polymorphisms (SNPs) currently known (Anzalone et al., 2019; Kantor et al., 2020).

Current applications

Unfortunately, this prime-editing technology is so recent that it is not yet possible to find extensive literature on it to corroborate such an ambitious statement. There is currently research being done using this gene-editing tool, but clinical trials are yet to be done. Nevertheless, it is already possible to find some interesting insights on its performance in different recent studies. For example, a study from 2020 by Kim et al. (Kim et al., 2020) has set the fundamentals for the practical application of prime editing. The study presents computational models for assessing the efficacy of a type of prime editor called PE2. The authors chose this particular PE among the four prime editors that have so far been developed (namely PE1, PE2, PE3, PE3b). The choice of not investigating all of them by comparing their individual efficacy lies on different facts. First of all, it is already known that the PE1 is the least efficient prime editor type, which makes it a poor candidate for future research use; secondly, PE2 actually is at the base of both PE3 and PE3b besides a concurrent sgRNA. Therefore, by investigating the efficacy of PE2 activity at any given target sequence, it is

possible to basically kill two birds with one stone, since predicting and evaluating the efficacy of PE2 will inevitably also affect the performance of PE3 and PE3b (Kim et al., 2020).
The closest to an *in vivo* research done using prime editing (PE) was published in 2020 and is reported in **Table 2**. This research sees the use of PE in adult stem cells grown in organoid culture models, and provides one of the first protocols for effective prime editing in human adult stem cells (Schene et al., 2020). The authors edited the gene *CTNNB1* associated with cellular survival. The desired edits to create mutations in liver- and intestine-derived organoid cells were successful, which shows that PE can be used in primary stem cells with similar efficacy and accuracy as observed in human cancer cell lines (Schene et al., 2020).
This is the first study to perform a whole-genome sequencing (WGS) analysis on prime-edited clones and their respective unedited control clones for assessing the fidelity level of prime editors applied to human cells. The result of the analysis indicates that there is no mutational fingerprint at the genome-wide scale left behind by the prime editors used. In addition, the safety of the prime editing technology utilized was also confirmed by absence of additional oncogenic mutations in tumor suppressors genes or oncogenes (Schene et al., 2020). Another important aspect of Schene et al. study is the results from the correction of disease-causing mutations. In order to investigate the functional correction potential of PE, the authors studied its effects on the deletion mutation of diacylglycerol-acyltransferase 1 (DGAT1) in patient-derived intestinal organoids. DGAT-1 is a DGAT enzyme expressed in intestinal epithelial cells and is involved in the absorption of dietary fats (Cheng et al., 2008; Schene et al., 2020). The mutation of DGAT1 results in lipotoxicity and cell death which are at the base of congenital diarrhea and protein-losing enteropathy (i.e. inflammation of the intestine) upon lipid intake by the patient. The DGAT1 mutation is characterized by the missing of 3-nt in exon 7 of DGAT1; thus the authors designed P3 plasmids to promote the insertion of the missing nucleotides. Ultimately they found that prime editing can repair small deletions with considerably higher precision and efficiency than the Cas9-initiated homology-directed repair (HDR) machinery, resulting in a higher ratio of correct editing to unwanted indels (Schene et al., 2020). In addition, the authors also performed a comparison of PE to base editing, which led to the conclusion that PE superiority lies only in correcting mutations that are uneditable by available base editors. Furthermore, the study confirms the therapeutic potential of this genome editor, thanks to the whole-genome sequencing (WGS) analysis results which suggest that prime editors do not induce genome-wide off-target effects (i.e. do not leave mutational signatures at the genome-wide scale). In fact, the desired edit was installed with 30-50% efficiency with a rate of 1-4% of unwanted byproducts at the pegRNA

or nickase sgRNA target sites. The absence of non-intentional oncogenic mutations of tumor suppressor genes or oncogenes confirms the safety of PE, and thus its great potential for future therapeutic use, above all when it comes to repairing human monogenic diseases (Schene et al., 2020).

The second study involving PE was done on *Drosophila* by Bosch et al. (2021) (reported in **Table 2**.). Based on this recent study, the germ cells can be edited using PE2 and concurrent pegRNA expression and the precise edit induced can then be transmitted from the founder generation to progeny (Bosch et al., 2021). Sequencing of editated G1and G2 flies using PE2 editing resulted in a high rate of transmission of desired edits to the progeny. At the genomic level the editing efficacy with PE2 and concurrent pegRNA expression was estimated to be between 10 and 40%; nicking sgRNA (PE3) was also used but although it led to higher editing frequency it also caused between 26 and 68% of indels frequency. Transgenic crossing was shown to result in higher transmission rates than injecting embryos directly. Additionally, it was demonstrated that in the male germline the transgenic expression of pegRNA together with sgRNA gave a higher transmission rate compared to the rate observed using embryo injection. Nevertheless, this approach has practical limitations. Generating transgenic pegRNA fly lines takes roughly one month, an aspect that makes the embryo injection approach superior to prevent delay germ-line editing experiments (Bosch et al., 2021).

4.1.3. RNA-directed editing: Aiming at the gene post-transcription instead of the gene itself

How it works

"RNA-editing" used to be identified as different biochemical and enzymatic processes that take place naturally in the cell. Evolutionary speaking, the animal nuclear RNA-editing systems originally developed from mononucleotide deaminases. Later, two larger gene families arose from these deaminases, including the APOBEC-1 and ADAR genes (Brennicke, 1999). In particular the latter encodes the double-stranded RNA-specific adenosine deaminase, an enzyme that is able to act on the RNA; this is also why the enzyme is called ADAR, an abbreviation that stands for a*denosine deaminases acting on RNA*.

The RNA modifications that result from these processes are important for regulating gene expression and the diversification of the resulting products. The programmed RNA-editing tool was developed based on these principles and targets the messenger RNA (mRNA) rather than directly in the DNA.

The impermanent nature of the RNA-editing is an advantage of the programmed RNA-editing tool over CRISPR/Cas9 and prime editing. This is an aspect that prevents permanent

off-target mutations to be made in the genome. Since the mRNA molecules are only transient within the cells, it is possible for this approach to be reversible and controllable during studies. The ADAR is able to catalyse the adenosine-to-inosine (A-to-I) (**Fig. 3**) editing on double stranded RNA (dsRNA) during post-transcriptional nucleotide modification of mRNA ("ADAR", NCBI, U.S. National Library of Medicine). Instead, the former gene family encodes for the APOBEC-1 protein (Apolipoprotein B mRNA-editing enzyme, catalytic polypeptide 1 ("APOBEC 1", NCBI, U.S. National Library of Medicine)), which is a C-to-U-editing enzyme (**Fig. 3**) heavily involved in antibody diversification and antiviral response (Christofi and Zaravinos, 2019).

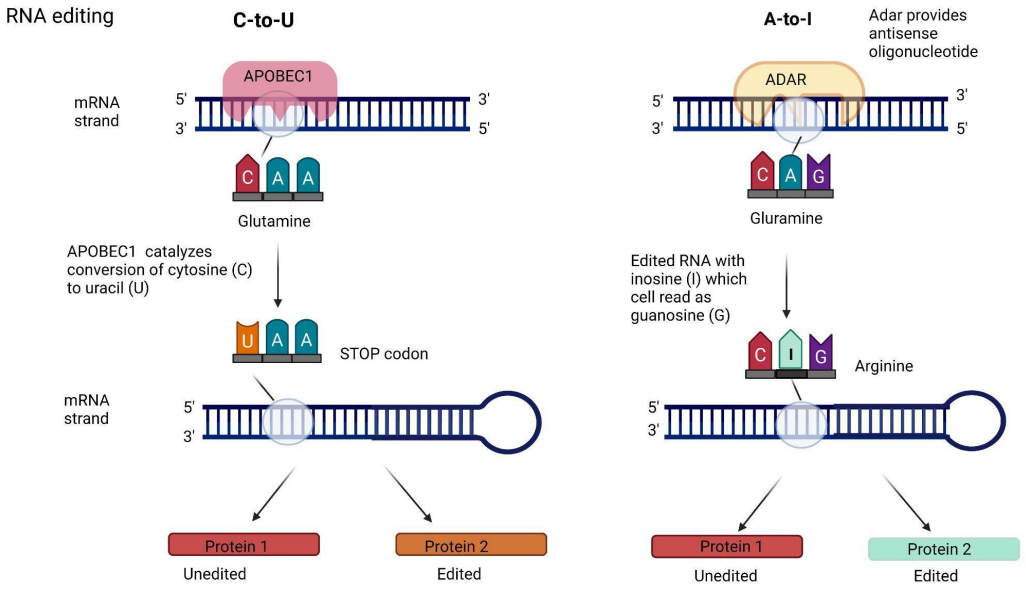

Figure 3. RNA-editing illustrated for both APOBEC1 and ADAR editing. In the case of APOBEC1 the cytidine deaminase is able to recognize on the mRNA strand the cytidine (C) base and to catalyze its conversion into uracil (U) changing the final protein. In the case of the ADAR, the adenosine deaminase is able to recognize the adenosine (A) base and to convert it into inosine (I) which is then read by the cells as guanosine (G). In this case too, the resulting protein will be different from the unedited one. Adapted from: "RNA-editing in the forefront of epitranscriptomics and human health" by Christofi and Zaravinos, 2019, *Journal of Translational Medicine,* 17: 319. Copyright © The Author(s) 2019. Created with BioRender.com

The A-to-I editing is a predictable type of RNA modification, since inosine is read as a guanosine in biochemical reactions; this is thanks to the fact that their structures are similar. Based on this notion, in 2011 Stafforst and Schneider hypothesized that it would be possible to induce similar RNA modifications by introducing exogenous ADAR enzymes tethered to gRNAs complementary to the target sequence (Stafforst and Schneider, 2012). They suggested that out of 20 canonical amino acids 12 can be targeted, including Asp, Glu, Asn,

Gln, His, Lys, Arg, Ser, The, Tyr, Ile, and Met/Start, as well as all three Stop codons. This makes it possible to manipulate RNAs and their protein products by artificially harnessing enzymatic A-to-I deamination. The challenge that they had to overcome was the infeasibility of directing ADAR enzymes at a new substrate for inducing a specific reaction. This is due to the fact that the extent at which A-to-I editing takes place at a particular site, depends on sequence context which follows precise rules, also referred to as "preferences" (Polson and Bass, 1994; Lehmann and Bass, 2000). However, they managed to overcome this obstacle by applying simple Watson-Crick binding rules. They created gRNA-deaminase conjugates by fusing isolated C-terminal deaminase domain of hADAR1 (a type of human ADAR which has a binding preference for 5′ nearest-neighbours such as U>A>C>G and for 3′ nearest-neighbours such as G>C≈A>U (Eggington et al., 2011)) to the C-terminus of a SNAP-tag domain (an engineered DNA repair protein -alkylguanine-DNA alkyltransferase). They also turned hADAR1 into a gRNA-dependent enzyme by removing the hADAR1 natural substrate-binding domains (Stafforst and Schneider, 2012). Thus, they were able to create a programmable enzyme that can target whatever RNA sequence (including mRNA sequences) one desires by designing appropriate gRNA for it. The RNA-editing enzymes are recruited by molecular tools and engaged by the site-directed RNA-editing to target sites. These enzymes enable the correction of G>A and T>C mutations. Since such mutations characterize most of all pathogenic point mutations in humans, this method could be promising for future therapeutic use (Fry et al., 2020). The main challenge of this technology concerns mostly the delivery method for transfecting to cells.

Current applications

The first study reported in **Table 2.** involving RNA-editing was supervised by one of the original developers of programmed RNA-editing, namely Stafforst. He has, with his team at the University of Tübingen, recently developed a novel precise RNA-editing which is able to recruit ADARs endogenously (Merkle et al., 2019). The reported novel method in this study, named RESTORE (recruiting endogenous ADAR to specific transcripts for oligonucleotide-mediated RNA-editing), is composed of engineered chemically optimized antisense oligonucleotides that are able to recruit endogenous human ADARs. The results of the study are promising and are based on effects observed in several different human cell lines (HeLa cells) as well as in human primary cells. The authors edited the GAPDH ORF in Hela cells with and without INF-α. It was estimated that with using INF-α the on-target frequency increased from 25% to 52%. All the off-targets detected were all in noncoding regions with a

frequency of between 17 and 55%. The study presents a fairly efficient and effective method where almost no off-target editing was observed. The results of this study are thus promising for treating inherited genetic diseases by targeting hepatocytes (Merkle et al., 2019).
The second RNA-editing study involved the use of a similar method to RESTORE, called LEAPER (leveraging endogenous ADAR for programmable editing of RNA) which employs short engineered ADAR-recruiting RNAs (arRNA) (Qu et al. 2019). LEAPER appears to be able to achieve up to 80% efficiency (**Table 2.**). Regrettably, despite its high efficiency, it is not as effective as RESTORE, since it induces off-target edits, although rarely.

Two studies have demonstrated for the first time robust results by using engineered RNA-editing in vivo. The two studies, both led by the same first author, present successful editing on mouse models for human diseases. In the first study (Katrekar et al., 2019) the team of researchers engineered and optimized two orthogonal toolsets for sequence-specific programmable RNA base editing for *in vitro* and *in vivo* use. In order to induce the editing, they developed a system for targeted RNA-editing via ADAR1/2 with associated adRNA (ADAR recruiting RNAs). However, the RNA-guided ADARs induce an ectopic expression, which has poor outcomes mostly due to their oncogenicity. On top of this, based on this study's results, general toxicity is also another severe side-effect (Katrekar et al., 2019). Overall this study was a foundation for the second one, which presents the use of an endogenous ADAR recruitment system in the IDUA-W392X mouse model of mucopolysaccharidosis type-I-Hurler (MPS I-H) syndrome. This study addresses and resolves two important problems: 1) the short half-life and residence times of guide RNAs, which if not resolved, makes the use of programmed RNA-editing as therapeutic unlikely; 2) the oncogenicity and toxicity due to use of exogenous ADARs. To solve the former, the authors engineered a highly stable circular ADAR recruiting guide RNAs (cadRNAs). For the latter, they developed and used an endogenous ADAR recruiter system. The study reports that *in vivo* on-target editing frequency was between 11% and 38% but the frequency of off-target effects is not clear, which in any case appears to be very low (Katrekar et al., 2021).

Table 2. Results summary for easier comparison

Biomedical engineering tool	**Delivery method/ study type**	**Cell types /Disease**	**(1) Off-target / (2) indels frequency**	**Editing efficacy**
CRISPR-mCas9	LNPs (LNP-INT01) *In vivo*	Liver hepatocytes/ amyloidosis in human (Finn et al., 2018)	(1) Possible post-editing indels generation in non immunocompromised subjects due to mCas9 (2) Not taken into consideration due to no risk associated	~70% efficacy for a single dose in rats
CRISPR-mCas9	LNPs (CLAN) *In vivo*	Intestinal macrophages /inflammatory diseases (Xu et al., 2018)	(1) Possible post-editing indels generation in non immunocompromised subjects due to mCas9 Less than 0.5% off-target frequency (2) 58.6% indels frequency	56.8% knockout efficacy for a single injection in mice
CRISPR/Cas9	Electroporation *Ex-vivo* editing Injection of edited cells directly in patients *Preclinical trials* ClinicalTrials.gov (ID: NCT03655678 for CLIMB THAL-111 and NCT03745287 for CLIMB SCD-121)	CD34+ hematopoietic cells/Transfusion-dependent beta-thalassemia (TDT) and sickle cell disease (SCD) (Frangoul et al., 2021)	No off-target nor indels detected	Patient 1 (TDT): Allelic editing frequency of 68.9% Patient 2: (SCD): Allelic editing frequency of ~80%
CRISPR/Cas9	Direct injection of organ	Leber congenital amaurosis (LCA), a	Not yet known	Not yet known

	Preclinical trial (ClinicalTrials.gov ID: NCT03872479)	retinal degenerative disease (ClinicalTrials.gov ID: NCT03872479)		
Prime editing (PE3)	Electroporation *In vitro organoids*	Liver- and intestine-derived organoids/ hepatocellular carcinoma/ other carcinomas and human monogenic diseases (Schene et al., 2020)	(1) No off-target effects or low rate of unwanted byproducts (2) No indels detected	30-50% editing efficiency rate
Prime editing (PE2+pegRNA and PE3)	Generation of transgenic flies *Drosophilia in vivo* models	Germ cells/ genetic heritable diseases (Bosch et al., 2021)	(1) PE2+pegRNA: no off-target effects detected (2) PE3: 26-68% indels frequency	PE2+pegRNA: 10-40% editing efficacy PE3: 2-4% higher editing frequency than PE2+pegRNA
Programmed RNA-editing (RESTORE)	Transfection agent + ASOs *In vitro* using primary cells and cell lines	Different cell lines and human primary cells/ hepatocytes targeted for inherited genetic diseases (Merkle et al., 2019)	(1) Between 17 and 55% off-target effects found only in noncoding regions. (2) No indels reported	Without INF-α: 25% on-target frequency With INF-α: 52% on-target frequency
Programmed RNA-editing (LEAPER)	Viral and non-viral vectors *In vitro* using primary cells and cell lines	Different cell lines and human primary cells/ hepatocytes targeted for inherited genetic diseases/ monogenic diseases (Qu et al., 2019)	(1) ~37% off-target editing frequency (2) No indels reported	~80% editing efficiency
Programmed RNA-editing (cadRNAs)	Viral vector *In vivo*	Liver/hepatocytes / Mucopolysaccharidosis type-I-Hurler (MPS I-H) syndrome (Katrekar et al., 2019)	(1) (2) Unclear (reported to be apparently almost 0)	Between 11% and 38% on-target editing frequency

Based on the reported results, all three tools are highly optimizable and can be used for different types of *in vivo* studies using different types of delivery systems. The results show

that the programmable RNA-editing tool optimization presents substantial increase in editing efficiency from one version to the other. This can be seen in the editing efficiency achieved by LEAPER compared to RESTORE which is rather significant. However, despite the better efficiency seen in all the different optimization versions of these tools, the optimizations often lead to a higher off-target editing frequency. In particular in the RNA-editing method, it appears that the low off-target frequency goes hand in hand with a low editing efficiency. This problem does not seem to affect the other methods that are based on the CRISPR technology.

4.2. Potential applications of gene-editing in atherosclerosis: Opportunities and challenges

Since CD36 has such an important role in atherosclerosis, and indirectly to chronic inflammation, it has become crucial to understand and to investigate this scavenger receptor. Many studies on animal models have shown how the genetic deletion of this PRR or the blockage of its signaling cascade can reduce atherosclerosis lesion formations and can represent possible valuable therapeutic strategies for future atherosclerosis treatment (Tian et al., 2020). Therefore, performing experiments that knock out or disrupt the expression of CD36 in human macrophages using genetic engineering tools (e.g. knocking out using CRISPR/prime editing or disrupt the CD36 protein with RNA-editing) can not only provide the basis to understand its regulation, tissue specificity, expression and pathogenic processes, but could also help develop precise and safe treatments for atherosclerosis patients. In addition, studying such receptors could shed more light on their additional importance in the normal physiological state of the cell, besides their already known roles (e.g. phagocytosis (Erdman et al., 2009), homeostasis, inflammation, lipid metabolism (Daviet et al., 1997) just to name a few. However, applying the different editing tools comes with its own set of challenges. For what concerns CRISPR/Cas9 and prime editing, the main challenge would be the off-target effects, which *in vitro* could lead to cell death (possibly due to disruption of other gene linked to cell survival), whilst in *in vivo* could lead to carcinogenesis or physiological disorders induced by unwanted gene mutations. Furthermore, the efficacy of the knockout could result too low to have a total effect on silencing the expression of the CD36 gene, both *in vitro* and *in vivo.* This would ultimately lead to difficulties in studying the receptor in the context of atherosclerosis. In a clinical setting these challenges would be severe enough to not allow the use of these tools for studying (nor treating) atherosclerosis. The RNA-editing application also presents its own challenge that could hinder the use of this

tool, both for *in vitro* and *in vivo*. Indeed, the RNA-editing effect on the CD36 protein would be only transient and not total (i.e. disrupting already transcripted proteins does not prevent the gene to keep producing more). It would be crucial to knockout the CD36 gene completely and permanently to actually be able to study what the short and long term of the knockout leads to. In a clinical setting disrupting the CD36 protein alone would not be useful to study a complex disease such as atherosclerosis.

4.3. gRNAs design and analysis of cell CD36 gene expression

gRNA design for CD36 target sequence

The following sequences were the final three sequences selected for designing the sgRNA for the CD36 gene knockout (the PAM sequence is in red):

GATTCCTTTCAGATTAACGTCGG (targets exon 10)

GAATCCGACGTTAATCTGAAAGG (targets exon 10)

TCATTTCTATCAGGCCAAGGAGG (targets exon 14)

These three sequences are ranked based on their likelihood to be effective in the knockout of CD36 gene using CRISPR/Cas9. Interestingly enough, the three sequences are quite similar.

These sequences were selected among hundreds based on results from three different web-based computational approaches, namely:

CHOCHOP (see Appendix 1.)
VBC (see Appendix 2.)
GPP (see Appendix 3.)
Comparison of all scores (see Appendix, 4.)

Final designed DNA oligos and primers for LentiCRISPRv2 gRNAs cloning

The following oligos are the final oligos designed for making gRNAs compatible with the lentiCRISPRv2 vector. In **Table 3.** there are reported the final oligos for the primers, while the final designed gRNAs oligos compatible with the lentiCRISPRv2 vector DNA are reported in **Table 4.**

Table 3. Oligos for primers designed based on gRNAs sequences

Target sequence	Oligos DNA
GATTCCTTTCAGATTAACGT CGG	ggatccTAATACGACTCACTATAGATTCCTTTCAGA TTAACGTGTTTTAGAGCTAGAAATAG
GAATCCGACGTTAATCTGAA AGG	ggatccTAATACGACTCACTATAGAATCCGACGTTA ATCTGAAGTTTTAGAGCTAGAAATAG
TCATTTCTATCAGGCCAAGG AGG	ggatccTAATACGACTCACTATAGTCATTTCTATCA GGCCAAGGGTTTTAGAGCTAGAAATAG

Table 4. Final gRNAs oligos compatible with lentiCRISPRv2 vector

Target sequence	Oligos
GATTCCTTTCAGATTAACGTCGG	CACCGGATTCCTTTCAGATTAACGT
	AAACACGTTAATCTGAAAGGAATCC
GAATCCGACGTTAATCTGAAAGG	CACCGGAATCCGACGTTAATCTGAA
	AAACTTCAGATTAACGTCGGATTCC
TCATTTCTATCAGGCCAAGGAGG	CACCGTCATTTCTATCAGGCCAAGG
	AAACCCTTGGCCTGATAGAAATGAC

Assessing expression of surface CD36 in undifferentiated U937 cells and differentiated U937 and THP-1 cells by flow cytometry

The results from the antibody staining show that U937 cells stained for FITC CD36 antibody at all concentrations of antibody given (**Fig. 5** and **Fig. 6**). The cells appeared to be clearly divided in two populations, one with alive cells and the other with dead cells. Although it was expected to observe different shifts for the different concentrations, the results showed similar levels of staining for the samples. This indicates that the lowest concentration of anti-CD36 antibody was sufficient to stain the undifferentiated U937 cells.

The results from gene expression assessment (**Fig. 7** and **8**) show that in both THP1 and U937 cells it is possible to detect the expressed surface CD36, although there are differences between the two cell lines CD36 expression level (**Fig. 7b., 7d.** and **8f., 8h**.). As seen in **Fig. 7** and **8**, both cell lines express CD36. The fluorescence intensity however, changes dramatically between THP-1 cells and U937 when comparing **Fig. 7d**. and **8h.**. It is observed that U937 cells express surface CD36 at a higher level than THP-1 cells. By comparing **Fig. 7b.** and **8f.** with **Fig. 7d.** and **8h.** one can affirm that expression is greatly upregulated after PMA treatment in both cells.
Therefore, the results indicate that the assay used is suitable for detecting surface CD36 and can be applied to determine sgRNA specificity and efficiency of CD36 knockdown.

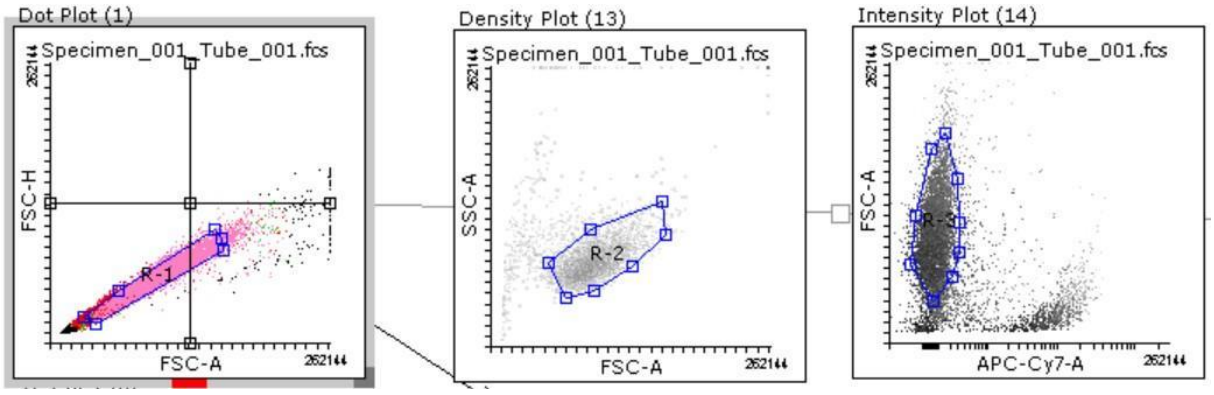

Figure 5. Gated singlets with gating strategy including 10000 cells in R-1.

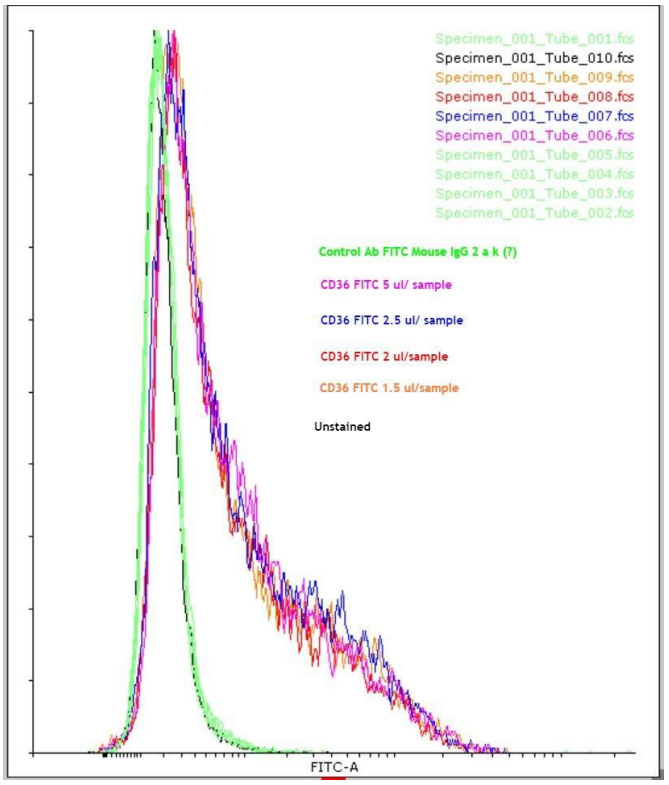

Figure 6. Overlapped histograms to show staining with antibodies of the different samples. The different shifts represent the different concentrations of antibody used to stain surface CD36. Each shift was obtained as a result of the gating strategy used. The green line represents the control cells stained with a different antibody than CD36 antibody. The black line represents the unstained cells. The different colours used represent the different concentrations of CD36 antibody used: Pink for 5µ/sample; blue for 2.5 µ/sample; red for 2 µ/sample; yellow for 1.5 µ/sample. The figure shows that the fluorescein isothiocyanate (FITC)-A mean fluorescence intensity for control and unstained cells are roughly the same, and that the fluorescence intensity of the different concentrations are similar.

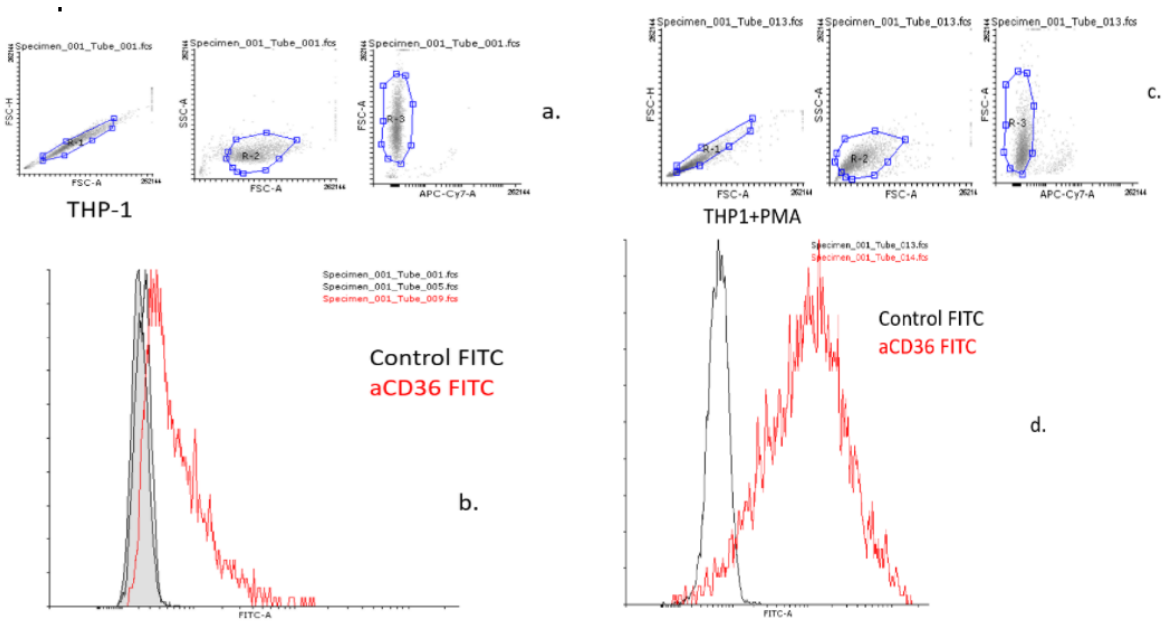

Figure 7. Figure legend for FACS scatter plots. THP-1 cells express surface CD36 and upregulate expression following PMA treatment. Comparison of expression between control CD36 FITC without antibodies and with antibody (aCD36 FITC) for undifferentiated THP-1 cells **(a.)(b.)** and for differentiated cells **(c.)(d.)**. Scatter plot for gated away doublettes (FSC-H vs FSCA), gated main population in SSC vs FSC (SSC-A vs FSC-A) and gated for cells negative for viability dye (FSC vs APC-Cy7) **(a.)(c.).** Left histograms **(a.)(f.)** show PMA-free cells treated only with fluorescein isothiocyanate (FITC) (black line) and PMA-free cells associated with a CD36 antibody (aCD36 FITC) (red line). Right histograms **(d.)(h.)** show PMA-treated cells with only FITC (black line) and PMA-treated cells associated with a CD36 antibody (aCD36 FITC) (red line)

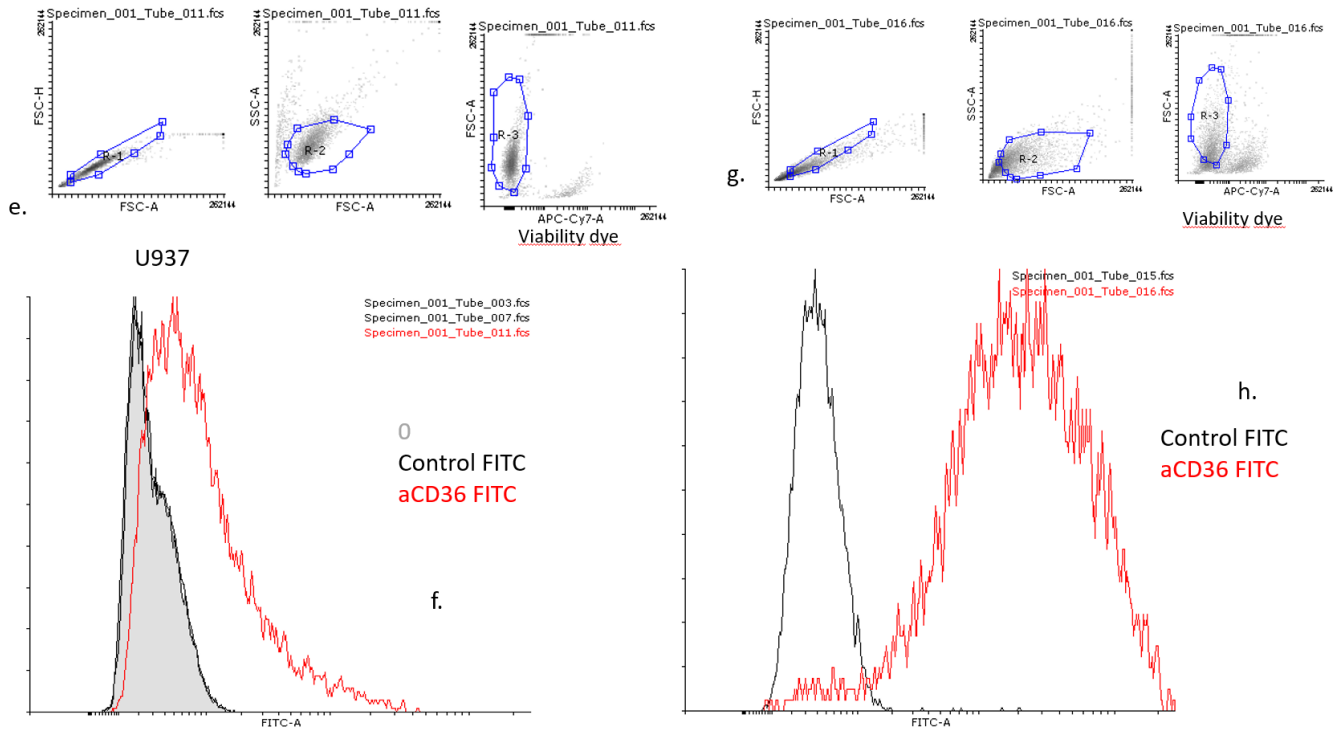

Figure 8. Figure legend for FACS scatter plots. U937 cells have high surface CD36 expression level even without PMA treatment.

Comparison of expression between control CD36 FITC without antibodies and with antibody (aCD36 FITC) for undifferentiated U937 cells **(e.)(f.)** and for differentiated cells **(g.)(h.)**. Scatter plot for gated away doublettes (FSC-H vs FSCA), gated main population in SSC vs FSC (SSC-A vs FSC-A) and gated for cells negative for viability dye (FSC vs APC-Cy7) **(e.)(g.).** Left histograms **(a.)(f.)** show PMA-free cells treated only with fluorescein isothiocyanate (FITC) (black line) and PMA-free cells associated with a CD36 antibody (aCD36 FITC) (red line). Right histograms **(d.)(h.)** show PMA-treated cells with only FITC (black line) and PMA-treated cells associated with a CD36 antibody (aCD36 FITC) (red line)

5. DISCUSSION

5.1. Biomedical narrative review: Insights into the main opportunities and challenges of applying CRISPR/Cas9, prime editing and RNA-editing in translational medicine

The future of medicine is unfolding in front of our eyes. The last few years have been a reminder of how fast medicine can progress. The excitement provoked by the novelty of CRISPR has quickly left space to leave a more bleak reality due to its dangerous off-target effects. Therefore, finding possible alternatives to DNA editing has become of paramount importance for finding new and better treatments for different diseases. The RNA-editing tool might have a second chance to shine as a possible equivalent to the CRISPR/Cas9 technology for medical use. The former has the big advantage of not disrupting the DNA of the patient, and yet to be able to post-transcriptionally modify the mRNA from the gene of interest, in order to disrupt the derived defective protein.

An article published in Nature (Reardon, 2020) suggested that CRISPR could soon be replaced by mRNA-editing. However, it is not clear in what particular settings this would be possible. The argument advanced by the author (that mRNA-editing is a safer technology than CRISPR, ergo it is a more promising therapeutics) is compelling, yet lacks a more in depth approach. The author fails to put side by side CRISPR/Cas9 and RNA-editing based on their performances in different biomedical research. In fact, there are no fact-based reasons that justify the author's suggestion about RNA-editing replacing the CRISPR technology in the article.

This is one of the motivations that has led to this thesis, where both technologies could have the opportunity to be presented transparently side by side. Given the broad subject and the limited amount of time, the review presented herein could not have been more exhaustive than it is. Therefore, the focus of the research was put only on the state-of-the-art of biomedical engineering editing tools, and thus only the most recent studies were taken into consideration. The search was also further narrowed to those studies presenting results from *in vivo* or *in vivo*-like research methodology (e.g. organoids). Unfortunately, the outcome of such an approach is greatly limited, and it leaves more unanswered questions than answered ones. Despite these limitations, some useful insights and future perspectives were gathered on the translational medicine potential that these three different tools hold.

Moreover, this thesis presents a model that can be used for ground research to better understand the role of CD36 in atherosclerosis. Developing similar models could help develop standard protocols that can easily be implemented and optimized by any biomedical researcher. Such an approach can make it easier to use these types of editing tools to study chronic inflammatory diseases/noncommunicable diseases, in light of the future perspectives that will be explored later on.

5.1.1. CRISPR/Cas9 as a tool to better understand and treat heritable blood disorders and diseases affecting immune privileged tissues and organs

The results herein examined give a promising picture for the use of CRISPR/Cas9 as a biomedical research tool and possible future therapeutics. The results are encouraging in particular for what concerns hematologic and ophthalmic diseases. However, in the first preclinical studies on Transfusion-dependent beta-thalassemia (TDT; CLIMB THAL-111) and sickle cell disease (SCD; CLIMB SCD-121) by targeting the *BCL11A* enhancer, despite the revolutionary aspect of the treatment, the trials present some problems that prevents drawing a proper conclusion on the potential of such a treatment as future therapeutics. First, the biggest limitation is found in the long-term effects, like the possible risks of post-alcylator myelodysplasia due to exposure to busulfan (Esrick et al., 2020). Second, given the fact that only a few participants were involved, and the results rely only on two different patients after one year from the treatment. Moreover, the researchers used an *ex-vivo* cell-based CRISPR/Cas9 genome editing technique, which involves the use of hematopoietic stem cells and progenitor cells obtained from healthy donors, and edited *in vitro* before being injected in the trial patients. This technique is quite well developed and has advantages over *in vivo* gene-editing, since it is easier to control and thus safer. Nonetheless, the problem of delivery to the cells still remains. In the study, electroporation is used as a delivery method. Hematopoietic cells are primary cells, and as such are already rather difficult to work with, compared to cell lines. Therefore, the use of electroporation comes with the downside of slowing down the progress of making a potential treatment. This could be one of the reasons why in the study of Fragoul et al. only two patients (among an unknown number of others) were reported to have been successfully treated. Future research could focus on using more effective and safe delivery methods; perhaps substituting the physical methods usually used in *ex-vivo* editing, with nanoparticles, in particular LNPs.

The other preclinical study (ClinicalTrials.gov ID: NCT03872479) for treating congenital blindness has yet to produce results, and the study is still in its early stages. However, it was decided to report it here due to its revolutionary delivery method. The delivery technique used in this study is the ultimate goal for what concerns future gene-editing treatments. Nevertheless, the transfection method can only be optimal on tissues similar to the eyes, which are known to be immune-privileged sites. This is due to the fact that in this case the gene-editing would be performed using a viral vector, which is an immunogenic agent. In the light of this, future studies could use a similar method to develop treatments for those diseases that are caused by genetic mutations found on other immune-privileged sites (e.g. testis).

5.1.2. Prime editing as a tool to better understand and treat carcinoma and heritable human monogenic diseases

In the first study taken into consideration, PE3 is used both for creating disease models, and to induce desired edit of disease-causing mutations. In the first part of the study, the authors edited the gene *CTNNB1*, which encodes for the beta‑catenin involved in cell survival signal pathways. Due to its important role in regulation and coordination of cell-cell adhesion and gene expression, its overexpression leads to the development of different types of cancers (MacDonald et al., 2009; Schene et al., 2020). Approximately 40% of hepatocellular carcinoma are found to present cells with carcinogenic mutations on *CTNNB1*. This particular aspect is crucial because it provides the opportunity to further study PE behavior in cells that better represent *in vivo* conditions, bringing this editing tool a step closer to proper *in vivo* investigations. The results gathered also underline the importance of testing various pegRNA designs in experiments. Furthermore, the results obtained from a PE treatment could theoretically also be transmitted to part of the patient's progeny, as seen in the *Drosophila* study by Borsch et al. (Borsch et al., 2021). Nonetheless, before reaching such a step at human level, it is of paramount importance to ensure first and foremost the safety of PE. Despite the fact that PE seems to be safer than other gene editors, as this gene-editing tool is more extensively studied there are unfortunately safety concerns arising. The first developers of the prime editing technology had reported how the efficiency of the edit can be boosted in mammalian cell lines by simply including the PE3 system (a nicking sgRNA) (Anzalone et al., 2019). However, a new study done on mouse embryos (Aida et al., 2021) have reported evidence that the PE double nick can cause indels at undesirable rates, surpassing that of the desired edits. These findings are somewhat confirmed by the Bosch et al. study in which similar high indel rates (this time in fly somatic cells) were also observed. Nonetheless, they

report that the use of the PE3 system in germ cells actually result in only 6-15% of edited progeny, which also contained indels or other off-target edits. There are severalt possible reasons for this. One hypothesis advanced by the authors is that "*germ cells could express different DNA repair components that lead to higher fidelity repair of double-nicking events"* (Bosch et al., 2021). Therefore, it would be advisable to use the PE3 system as a booster of prime editing efficiency in the germ line rather than in somatic cells. However, additional findings in the study of Aida et al. also suggest that "*sequential double-nicking with PE3b eliminates undesirable outcomes"*, but this type of PE system is only applicable for a fraction of edits (Aida et al., 2021). Therefore, future research should focus on studying the different DNA repair behavior in both the somatic and germ cells when PE3 is used, in order to better understand the dynamics that lead to more undesirable outcomes in one and not the other. Moreover, more research should be done to optimize the current PE types and to develop new types that do not induce undesirable outcomes, and are as effective as (if not more effective than) the current ones that are available. As in the case of CRISPR/Cas9, it should be a priority to develop a safe nonimmunogenic delivery method for the prime editing injectable solution. Tailored lipid nanoparticles are in this case also promising, and future research should further explore methods of developing optimal LNPs for possible therapeutic use of PE.

5.1.3. Programmed editing of RNA as a tool to better understand and treat point-mutation induced monogenic diseases

Although revolutionary, Stafforst's invention did not manage to create much noise, probably due to its inability to be delivered *in vivo* using viral vectors, something that the developers of CRISPR/Cas9 made possible almost immediately. Nevertheless, the fundamental role Stafforst and Schneider played in the development of a new kind of biomedical technology is undeniable. Despite the excitement around CRISPR/Cas9, the interest in RNA-editing has not died out. Quite the contrary, actually. In the last decade there has been a steady building up of better methods and protocols for bringing this editing tool into the clinic. For example, new tethering techniques have been replacing the original SNAP-tag (Stafforst and Schneider, 2012) with Lambda-N protein and box B element RNA (Montiel-González, 2016), and more recently with the MS2 system (Azad et al., 2017; Bhakta et al., 2018).

The main problem of RNA-editing is the ectopic expression of ADAR. This aspect makes it challenging to use it for development of therapeutics. The risk of inducing overexpression of the enzyme ADAR, has been reported to confer oncogenicity by aberrant hyper-editing of the

myeloma transcriptome (Teoh et al., 2018) and other human cancers. For example, it was newly discovered that the *ADAR1* overexpression alters the thyroid cancer cell aggressiveness as a consequence of the ADAR1-mediate A-to-I editing pathway (Ramírez-Moya et al., 2020). Since the problem seems to concern the ectopic-induced expression, the study results from the RESTORE method (Merkle et al., 2019) seems to indicate a possibility to recruit ADARs endogenously and still keep the RNA-editing precise. The main limitation, and at the same time the strength, of the study lies in the delivery method of RESTORE, which is done using a common transfection agent. The limitation is due to the fact that this transfection agent can be used only for *in vitro* experiments. Even so, this is also a strength because this agent is developed based on lipid nanoparticle principles, an aspect that could make it easier to develop future optimizations more oriented for *in vivo* experiments. However, this optimization could prove rather challenging to achieve, given the fact that RESTORE is heavily dependent on complex chemical modification of chemosynthetic antisense oligonucleotides (ASOs). Despite the fact that the ASOs compound is highly specific in their targeting, its delivery is quite challenging because it is possible only in specific organs (e.g. direct injection in spinal column) (Sheridan, 2021). However, new therapeutic drugs such as GalNAc (N-acetylgalactosamine) (Debacker et al., 2020) are being designed for targeted delivery of oligonucleotides to liver hepatocytes which gives hope for future use of an optimized version of RESTORE in a clinical setting. The other study by Qu et al. (2019) seems to point to a better method for RNA-editing, which increases its potential for future clinical use. The method, LEAPER, is able to recruit endogenous ADAR by employing short engineered arRNAs (ADAR recruiting RNAs). These types of RNAs can recruit native ADAR1 and ADAR2 enzymes for changing a specific adenosine to inosine. The method appears to be better mostly because it has more optimization potentiality than RESTORE, thanks to the fact that the arRNAs of LEAPER can be generated in different ways, including delivery by plasmid, chemical synthesis, and direct expression *in vivo* from viral and non-viral vectors. Regrettably, despite its high efficiency, it is not as effective as RESTORE, since it induces off-target edits, although rarely. The results of the Qu et al. study, although promising, are limited by the fact that they have not yet been challenged by testing *in vivo*. In addition, despite the fact that the authors have demonstrated the non immunogenicity of LEAPER itself in target cells, it does not mean that the risk of immunogenicity can be completely avoided. First of all, because the delivery systems used in the study and possible instability of the proteins involved are inherently immunogenic when used *in vivo* (Jiskoot et al., 2012); second of all, the immunogenicity was only tested based on possible mRNA

induction of interferon-beta (INF-beta) or interleukin-6 (IL-6). However, the triggering of the innate immune system by RNA duplex is just too broad and complex to be sure of absence of any innate response, since the presence of RNA duplex signals an ongoing viral replication in the cell. Therefore, future research should investigate in more depth the interaction between the arRNA in LEAPER and the innate immune system. Moreover, the behavior of the latter should be observed in *in vivo* studies for assessing the claims of the study on the non immunogenicity of the RNA-editing product as a whole. Despite the better efficiency than RESTORE observed in the optimization version LEAPER, more off-target editing frequency is also observed. This is an important aspect that has to be worked on, perhaps in the next optimization process. Unfortunately, it appears that low off-target frequency goes hand in hand with a low editing efficiency as well. Interestingly enough, this problem does not seem to affect the other methods taken into consideration and that are based on the CRISPR technology.

The studies led by Katrekar (2019; 2021) represent an interesting example of gene-editing tool optimization. Despite the promising results of this latest study, both have great limits; in part due to the high frequency of off-target editings induced by hyperactive editase that were observed, and in part due to the total disregard of possible immunogenic effects of the treatment. Future research should work on optimizing Katrekar's RNA-editing system to make it more effective and efficient; additionally, thorough investigations should be done to study possible immunological effects on mouse models of human diseases in which the cadRNAs editing system is used. As in the case of CRISPR/Cas9 and PE, it should be a priority to develop a safe nonimmunogenic delivery method for the programmed RNA-editing injectable solutions. Tailored lipid nanoparticles are in this case also promising, and future research should further explore methods of developing optimal LNPs for possible therapeutic use of this editing tool. Despite the challenges of RNA-editing systems so far discussed, post-transcriptional genetic editing is a promising clinical use of RNA-editing technology. However, in order to make substantial progress in the field of engineered RNA-editing tools, it is also crucial to develop new and better bioinformatics tools to better select editing sites.

5.1.4. Nanoparticles as better delivery systems than electroporation and lentivirus vectors for biomedical applications

The results gathered herein suggest that the use of nanoparticles (specifically LNPs) is a promising delivery approach that increases the potential of these editing tools in translational medicine. These nanoparticles not only preserve the efficacy of the editing, but also help

reduce off-target frequency by improving the editing precision. The astonishing power of these biomaterials used as non-viral carriers reside in the fact that they can be engineered in unbelievable ways in order to increase their delivery efficiency, and at the same time minimize their toxicity. In the Chen et al. study (Chen et al., 2017), a type of LNP is successfully used *in vivo* for inhibiting tumor growth and improving tumor-bearing mouse survival. However, despite its tumor-inhibition efficacy, the treatment is unable to eliminate solid tumors, above all if the tumoral cells happen to be in bulk. Therefore, the treatment alone would be useless in a clinical setting, but it could make a difference if paired with other standard oncological treatments. For example it could be used after surgical tumor mass removal to inhibit relapse. A big limitation of this study is that it does not investigate the off-target effects of the treatment, although it acknowledges it as a possibility. If the off-target factor is not included in a study, the treatment is doomed to never enter the clinic. The off-target effects are of paramount importance when a potential gene therapy is being studied, because the treatment can be successful in treating the disease, but solving one disease could signify the triggering of hundreds of others simultaneously.

The use of electroporation is promising for ex-vivo studies, and viral vectors are still the best option for successful in vitro experiments

Ex-vivo editing using electroporation as a delivery method also gives promising results, but it is a technique that could lead to more slowed down treatments due to the problems associated with the method (e.g. high cell death during transfection). Furthermore, electroporating *ex-vivo* is a methodology that can be used for few medical conditions, further limiting the use of this delivery method as part of possible future medical treatments.

The use of viral methods for delivery also seems to give promising results. The use of lentivirus as a vector for CRISPR/Cas9 has the advantage to induce the wanted genetic editing more easily than other methods. This is optimal for experiments done *in vitro*, but its immunogenic nature (Sakurai et al., 2007; Milone & O' Doherty, 2018) makes this type of delivery system not optimal for clinical use. Moreover, it appears to not help with the delivery challenge for the programmed RNA-editing tool either. This is because the usual adeno-associated virus vector used for cell delivery has limited cargo size (~4.5 kilobases), which makes the accommodation of both the protein and the gRNA challenging, since together they usually exceed the cargo load limit (Qu et al., 2019).

However, the use of viral vectors as delivery methods could be appropriate and effective in case of injection to non-immunogenic sites, for example for treatment in tissues and organs

that are immunologically privileged. A clear example is represented by the preclinical trial performed in patients affected by Leber congenital amaurosis (LCA) which received, with an intravitreal injection, a CRISPR/Cas9-based treatment containing an adenovirus as a main delivery vector. The treatment was successful and no adverse immune reactions were observed (ClinicalTrials.gov ID: NCT03872479).

Regrettably, in most of the studies taken into consideration, the disregard of possible adverse immunological effects from each tool is quite worrisome. Given the fact that these studies were designed for *in vivo* use, the immune response should be a high priority concern, as much as the other parameters taken into consideration (e.g. high efficacy *etc.*). This is of paramount importance above all for what concerns the study of inflammatory diseases, where the immune system is already severely compromised.

5.2. Possible applications of biomedical engineering editing tools: Future perspectives

Modern medicine is inexorably progressing towards more precise and individual treatments. Therefore, there is a growing need to develop novel ways to not only understand the underlying factors behind chronic inflammatory diseases, but also to be able to manipulate such factors that cause or even predispose to them.

Sterile inflammation as a source of chronic inflammation and cancer

Although inflammation is useful for fighting pathogens and for injury recovery, if it is not timely resolved, the permanence of the inflammation process in the body can lead to chronic systemic inflammation (Netea et al., 2017).

In the early 2000s, it was already well known that low-grade chronic systemic inflammation - which is generally characterized by high levels of C-reactive protein (hsCRP) in the blood - is associated with cardiovascular diseases and poor clinical outcomes (Ridker et al. 2000; Ridker 2003). Thus, in some cases chronic inflammation can be the source of different secondary diseases (e.g. diabetes mellitus, chronic kidney disease etc. (Furman et al., 2019).

Moreover, persistence of a state of chronic inflammation is associated with DNA damage, caused in part by reactive chemical mediators of inflammation, and in part due to nitrosative deamination, oxidation and halogenation reactions that take place with DNA (and RNA). The latter process is due to the infiltration of macrophages and neutrophils in the injured tissue, which triggers the generation of nitric oxide, superoxide and other reactive oxygen and nitrogen species (Lonkar and Dedon, 2010).

The persistence of inflammation can also lead to a vicious circle of interaction between chronic inflammation and cancerous cells. Chronic inflammation can promote cancerogenesis due to its role in mutagenesis (e.g. DNA damage induced by oxidative stress). Therefore, the regulation of inflammation is crucial for the state of the tumor microenvironment (Grivennikov et al., 2010).

Understanding the endogenous factors that lead to these immune dysfunctions is a crucial step, and biomedical research has been making continuous new discoveries with even the most traditional research tools. However, here is where biomedical engineering tools differ from past research methods: They can give us not only more information on these diseases, but at the same time, could also provide possible solutions to treat these complex diseases. Traditionally, the solution for chronic inflammation has mostly been found in

pharmacological means (e.g. anti-inflammatory drugs). Regrettably, despite their relative effectiveness in the short-term, this traditional solution can only give temporary relief of symptoms to the patients, but cannot resolve the root cause. It is also worth mentioning that these drugs do not come without side-effects, which sometimes can be rather dangerous (e.g. Non-steroidal anti-inflammatory drug induced gastrointestinal and cardiac toxicity) (Fries et al., 1991; Howard et al., 2007). Since different genetic engineering methods are currently available, valid alternatives to these drugs could be limitless. Yet, not all solutions would be optimal. This is why for each clinical situation there should be different genetic engineering methods used, tailored not only on the specific disease pre-existing risk factors or current pathophysiology, but also to the specific patient.

5.3.1. Some molecular disruptions behind the most common IMIDs that could be targeted and future perspectives for biomedical research and novel treatments using CRISPR/Cas9, prime editing or the programmed RNA-editing biomedical engineering tools

In the coming sections there are examined a few possible future perspectives for further basic biomedical research on some of the most common inflammatory diseases, and for the possible use of the biomedical engineering editing tools discussed earlier on.

i.. Rheumatoid arthritis

ii. Type 1 diabetes

iii. Multiple sclerosis

iv. Neurodegenerative disease of aging

v. Psoriasis

The focus will be put on those molecular mechanisms where targets can be identified for modifications by either CRISPR/Cas9 editing, RNA-editing or prime editing technologies.

The following future perspectives are based on grossly simplified underlying mechanisms of the inflammatory diseases taken into consideration. Therefore, the suggested perspectives are to be taken as mere examples of targets similar to CD36 that can be used in a similar experiment setups as the model previously presented.

<u>i. Rheumatoid arthritis:</u> Using CRISPR/Cas9 or prime editing for disruption of defective HLA-DRB1 and RNA-editing for post transcriptional arginine disruption

The most relatively common chronic inflammatory disease is rheumatoid arthritis (RA). The development of this autoimmune disease is correlated with certain human leukocyte antigen (HLA)-DR haplotypes. In particular, the "shared epitope" known as HLA-SE presents on the HLA-DRB1 gene; therein, a common amino acid sequence at position 70-74 was shown to be associated with the disease (Gregersen et al., 1987; Volkov et al., 2019). RA is also characterized by the production of autoantibodies such as rheumatoid factors (RF) and anti-citrullinated protein antibodies (ACPA), which are circulating before the onset of the disease. HLA-SE alleles activation and the ACPA response in RN patients are strongly linked, since it has been shown that HLA-SE alleles are only associated with ACPA positive RA (Huizinga et al., 2005; Volkov et al., 2019). Therefore, the HLA-DRB1 gene could be a strong candidate to be the target of interest. Disrupting this particular shared epitope expression could further our understanding of the relation between its expression and ACPA level in RA. If the two were shown to be positively correlated, future treatments could either be tailored on the specific HLA-DRB1 for genetic modification (e.g. using CRISPR/prime editing technologies) or on the disruption of posttranscriptional modification (citrullination/deamination) of arginine (e.g. using RNA-editing technology), thus preventing the engagement of ACPA which are activated by recognition of citrulline in proteins.

<u>ii. Type 1 diabetes:</u> Using CRISPR/Cas9 or prime editing for INFα receptor engineering and RNA-editing for post transcriptional modification of beta-cells produced protein

Type 1 diabetes (T1DM) is another relatively common autoimmune disease, above all in pediatric age. T1DM is characterized by several different innate immunity and inflammatory mediators of which sinergy contributes to early induction and amplification of the immune response attacking beta-cells, thus leading to their suppression and ultimately death. Most genes expressed by beta-cells (more than 80% of the identified candidate genes) produce protein products involved in the antiviral response that triggers the innate immune response. In particular, the exposure of beta-cells to INFα (proteins mainly involved in antiviral immune response) induces several of the hallmarks observed in islets of T1DM patient's pancreas.

It has been shown that the decreasing of the expression or function of TYK2 (a kinase involved in the transduction of downstream signalling from the INFα receptor) results in a protective effect against T1DM. Therefore, by preventing excessive inflammatory response

provoked by the antiviral response, it might be possible to decrease the risk of T1DM (Op de Beeck and DiLorenzo, 2016; Eizirik and Colli, 2020). A recent preclinical study by de Brachène et al. (Brachène et al., 2020) has shown that by using TYK2 inhibitors successfully decreased the pro-inflammatory and pro-apoptotic effects of INFα on beta cells, without sensitizing them to viral infection. Although this approach seems promising, the problem of a beta-cells produced protein still remains, thus it can still be a source of inflammatory response. Therefore, a step further could be taken by either directly genetically engineering the INFα receptor (e.g. by using CRISPR/prime editing technology) in order to make it to unresponsive to the beta-cells produced protein products that trigger the receptor in the first place, or by post transcriptionally modify these particular protein products (e.g. by using RNA-editing). Thus future research should further study such a strategy in order to find out if it might be able to stop these byproducts from ligating to the INFα receptor all together.

***iii. Multiple sclerosis:* Using CRISPR/Cas9 or prime editing for modification of ICAM5 gene. RNA-editing inapplicable.**

Multiple sclerosis (MS) is an autoimmune and neurodegenerative disease characterized by chronic inflammation which causes changes such as demyelination, axon loss, cortical and deep gray matter damage as well as atrophy. In an experimental model for MS, named experimental autoimmune encephalomyelitis (EAE), it was observed that neuroinflammation and demyelination are preceded by increased expression of ICAM-1, VCAM-1, and MAdCAM-1 by the choroid plexus (CP) epithelium. In addition, leukocytes are recruited on the site, and focal necrosis of individual epithelial cells take place (Engelhardt et al., 2001; Monaco et al., 2020). In progressive MS, neurodegeneration at cortical and subcortical locations present different molecular mechanisms from those observed in active demyelination. In particular, mechanisms associated with the inflammatory molecular load of the cerebrospinal fluid are correlated with a specific widespread pattern of neurodegeneration. Damage in the gray matter and the inflammation of the cerebrospinal fluid (CSF) in multiple sclerosis, both involve a dysfunction of the blood-cerebrospinal fluid barrier and inflammation in the choroid plexus (Monaco et al., 2020). In MS the resolution of inflammation fails due to disruption of adhesion molecules such as the intercellular adhesion molecule 5 (ICAM5) which are present in the cerebral and hippocampal neurons. The extracellular domain of ICAM5 is cleaved and released into the CSF and blood, where it stimulates the expression of anti-inflammatory cytokine IL-10, modulates the synthesis of proinflammatory cytokines, and represses T cell activation (Paetau et al., 2017; Celarain and

Tomas-Roig, 2020). A study on patients with primary progressive MS (PPMS) with non-recoverable demyelination and degeneration (Kulakova et al., 2016) showed that the patients presented higher methylation levels for ICAM5 than patients with relapsing-remitting MS (RRMS), suggesting that the overactivation of inflammatory response in MS might be due to aberrant methylation pattern of particular anti-inflammatory genes (Celarain and Tomas-Roig, 2020). Another more recent study (Birkner et al., 2019) confirms the neuroprotective role of *ICAM5* in MS progressive neurodegeneration. In the study, *ICAM5* knockout mice showed a more severe EAE disease course; yet, when shed soluble form of ICAM5 (sICAM5) was administered intrathecally (i.e. injection between layers of tissue that covers the spinal cord), an amelioration of EAE disease symptoms was observed. In human patients with PPMS the cerebrospinal fluid has decreased level of sICAM5. Therefore, future treatments could see targeting the gene that codes for ICAM5 in the telencephalic neurons of the central nervous system, by using CRISPR/prime editing technology. Future research could use plasmids such a the Tet1 demethylase created by Ronggui Hu's laboratory (Xu et al., 2016) (the pdCas9-Tet1-CD plasmid) for observing the effects of decreasing methylation and activating transcription of the *ICAM5* gene, and thus progress the path towards a possible novel treatment for MS. In this case, the use of RNA-editing would not be useful since the problem with *ICAM5* is not the coding of a defective protein, but rather a low expression of the gene due to hypermethylation of it.

<u>iv. Alzheimer's disease:</u> Using CRISPR/Cas9 or prime editing for NLRP3 inhibition and RNA-editing for post transcriptional modification of the NGAL protein

Alzheimer's disease (AD) is one of the most common types of dementia among people over 65 years of age and it is characterized by cognitive decline and memory loss. As in the case of MS, the dogma of immune-privilege sites of the nervous system is also challenged in the case of AD. It has been shown that neutrophils, together with the subsequent amyloid plaques formed, may be able to translocate and co-localize within the brain parenchyma through the cerebral blood vessels. Moreover, the dislodged neutrophils and plaques can adhere in the cerebral small vessels (Bawa et al., 2020; Cruz Hernández et al., 2019). This neutrophils conglomeration is then able to mediate the activation of the innate immune system in the brain. The activation of neutrophils leads to inflammatory response and the generation of reactive oxygen species (ROS) which have been shown to be associated with AD pathology in humans (Bawa et al., 2020; Pietronigro et al., 2017; Dong et al., 2018; Vitte et al., 2004). Furthermore, their adhesion to the brain capillaries reduces cortical blood flow, possibly

impairing normal memory function, as shown in recent developed AD mouse models (Cruz Hernández et al., 2019). The most relevant marker for inflammation found postmortem in AD patients' brains is lipocalin-2 (NGAL), a pro-inflammatory molecule which is selectively secreted in neutrophils granules, which is found in high levels in patients with AD (Bawa et al., 2020; Naudé et al., 2012; Dekens et al., 2017). Whilst increased levels of NGAL in plasma has been correlated with cognitive decline (Bawa et al., 2020; Choi et al., 2011), in particular with the decline of executive function (Bawa et al., 2020), memory loss appears to be mediated by the autophagy impairment induced by caspase-1-dependent inflammation in response to beta-Amyloid peptide accumulation. Thus, the block of autophagy allows the beta-Amyloid peptide to form plaques typical of AD (Álvarez-Arellano et al., 2017).
Since inflammation has been suggested to affect AD patients mood, memory, and other cognitive domains (Hall et al., 2013; Álvarez-Arellano et al., 2017; Holmes C. et al., 2011; Swardfager et al., 2010; Lai et al., 2017), it would be desirable to properly research the effects on these different domains on a clinical level. Future research could use engineering editing tools to disrupt the NGAL protein release in the brain (e.g. using post transcriptional modifications by RNA-editing) to observe the effect on the executive functions, and to prevent autophagy blockage due to the hyperactivation of the caspase-1-dependent inflammation. Another approach could be inhibiting the formation NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3) inflammasome by knocking down the *NLRP3* gene (e.g. using CRISPR/prime editing targeting the NLRP3 gene) to observe the effects on memory loss. If such research confirmed the hypothesis of the role played by inflammation on AD, optimization of these experimental interventions could become possible effective future treatments.

***v. Psoriasis:* Using CRISPR/Cas9 or prime editing for modification of Card14 and RNA-editing for post transcriptional modification of the CARD14 protein**

Psoriasis is a relatively common chronic dermatological disorder, characterized by thickening and inflammation of the epidermis where keratinocytes are hyperproliferative. In addition, substantial dermal infiltration of T cells is also observed. However, the main driver of the condition is the type 17 cytokines (IL-17), released by activated skin-resident dendritic cells (DCs) which are thought to orchestrate the pathogenesis of psoriasis. Thus, the keratinocytes are considered to be merely executioners of the released cytokines IL-17 and IL-22. Despite the increased understanding on the matter, it is still debated if the onset of psoriasis is due to the dysregulation of the innate immune system or due to the genetic predisposition to

keratinocytes hyperproliferation to which the immune system reacts (Zhang et al., 2021). Nevertheless, new evidence has been pointing more towards the keratinocyte having a more pivotal role in the development of psoriasis. The identification of the gene *Card14*, which codes for the CARD14 protein, has led to a great breakthrough on the matter (Tsoi et al., 2012; Zhang et al., 2021). The CARD14 protein expression is expressed at high levels in skin keratinocytes in both healthy and lesioned skin. Missense mutations have been identified in the coding sequence of *Card14* gene. Different mutations have been discovered to be associated with different types of psoriasis (e.g. psoriasis vulgaris, generalized pustular psoriasis, pityriasis rubra pilaris, and atopic dermatitis). The mutations induce skin acanthosis (i.e. overgrown stratum spinosum of skin) and increase immune cells infiltration in skin (Mellett, 2020; Zhang et al., 2021). These are gain-of-function mutations which induce the expression of psoriasis-associated pro-inflammatory genes, and they have been shown to induce psoriasis-like skin inflammation *in vivo* (induced TNF-dependent skin and systemic inflammation) (Manils et al., 2020; Wang et al., 2018; Mellett et a., 2018; Sundberg et al., 2019; Van Nuffel et al., 2020; Zhang et al., 2021). In a recent study by Zhang et al. (2021) heterozygous and homozygous mice harboring $Card14^{E1384}$(known as a psoriasis-associated mutation) or $Card14^{\Delta E138}$ could spontaneously develop extensive skin and systemic inflammation. Therefore, future treatments could be developed using genetic engineering tools to either silence the psoriasis-associated mutations of *Card14* (e.g. using the CRISPR/Cas9/ prime editing technology) or even modify post-transcriptionally the CARD14 protein expressed by $Card14^{E1384}$ in keratinocytes since the missense mutation E138A is a point mutation (e.g. using the RNA-editing tool). This protein correction could prevent the induction of the TNF-dependent skin and systemic inflammation observed by Manils et al. (Manils et al., 2020).

5.4. Limitations of the biomedical narrative review

The biomedical narrative review herein presented is only a qualitative snap-shot of the most current studies, and it is not as exhaustive and methodological as a systematic and meta-analysis would be. In addition, some most recent developments in the biomedical field have not been taken into consideration, due to lack of time, and because it was decided to prioritize the presentation of a more diverse group of novel editing technologies.

In this regard, it is relevant to mention a type of RNA-editing technology which uses the CRISPR technology: CRISPR/Cas13. This new editing tool has been used and optimized since 2016 showing to be the most promising RNA-editing approach for its seemingly more precise and efficient targeting and editing. In particular the Cas13a version of this technology has been utilized successfully for transcription regulation and other biotechnological engineering (Abudayyeh et al., 2016; Cox et al., 2017; Gootenberg et al., 2017; Gootenberg et al., 2018; Konermann et al. 2018; Myhrvold et al., 2018; Terns, 2018; Li et al., 2020). Despite the role this tool has recently been playing in RNA-editing, it was decided to not include this alternative editing for RNA, mostly due to the longer permanence the Cas13 nuclease exhibits, which represents the biggest problem of this method. The safety concerns that result from this excessive and prolonged exposure are mostly based on the higher chance for undesirable RNA mutations and other significant off-target effects. However, this problem is being currently addressed by Li et al. (2017) who have designed and tested a "Cas13 off-switch" method that could pave the way for improving gene-editing for eventual clinical applications also for CRISPR/Cas9, by reducing possible toxicity and non-specific targeting of the system (Li et al., 2017).

Beside the reason aforementioned, it was also decided to leave this novel tool behind in favor of giving more space to non-CRISPR solutions, thus giving a broader picture of the state-of-the-art of biomedical engineering editing that goes beyond CRISPR.

5.5. Model for CD36 gRNAs design for using CRISPR/Cas9

The importance of designing good gRNAs

In order for a CRISPR/Cas9 knockout to be successful, the very first step to take is to design good single guide RNAs. This step allows for the precise localization of the target of interest by providing a complementary sequence to the target sequence. The more precise the gRNA design, the easier it is for the Cas9 nickase to individualize where to perform the correct double strand cut. This can ensure less off-target effects and high knockout efficacy. When designing gRNAs to be used in an experiment, it is crucial to research that such guides are not compatible with other gene sequences before testing them. In case some other gene has a similar gene sequence to that of the target of interest, it would be crucial to first and foremost study what role this similar gene plays in the cell and in the organism. This is why it is important to research the knockout effects of CRISPR/Cas9 first *in vitro* and then *in vivo* in order to observe the different roles played by a same gene, and what disrupting it would signify for the cell and the organism normal function and well-being. Moreover, the good design of gRNAs is crucial also for prime editing, because the pegRNA rely on the gRNA to find the right DNA sequence to modify after the Cas9 single strand cut has been performed.

The importance of choosing the best cells for gene-editing

THP-1 cells and U937 cells are both suitable cell lines for CD36 knockout above all for testing the quality of gRNAs. However, since the U937 cell line has a high level of CD36 expression even if the cell is undifferentiated, it would be the best choice for the ultimate analysis of knockout of CD36 in the cell. In fact, being able to knock out undifferentiated cells can give the possibility to observe if the knockout remains also once the cell differentiate. In addition, it can also give more insight on the effects the knockout has on the cell functions on both differentiated and undifferentiated cells. However, the selection accounts only for what concerns the analysis of gene expression. Therefore, it might be a good model cell for testing the CRISPR method used, but it might not be the ultimate best choice for further studies on atherosclerosis. In addition, CD36 plays different roles in the physiological state of the cell (Erdman et al., 2009; Daviet et al., 1997). Thus it can be quite challenging to try to knock out CD36 both *in vitro* and *in vivo*. First and foremost, because the

process of knocking out a gene includes the transfection of CRISPR to the cells, which is per se quite challenging. As mentioned previously, the delivery of gene-editing tools is indeed one of the main challenges of these new biomedical engineering technologies. In this sense, the choice of cells becomes crucial, because cell lines are easier to work with than primary cells when it comes to transfection (ThermoFisher, "Factor Influencing Transfection Efficiency"). Thus, this may be yet another reason to use U937 cell lines. However, even if the transfection part was not a problem, knocking out the receptor and keeping the cell alive long enough to analyse the knockout could also be a big problem. Above all for what concerns *in vitro* studies. This is because, while *in vivo* the disruption of CD36 in the cell could be somewhat compensated by cell survival signaling coming from other surrounding different cell types (Shilo and Schejter, 2014), which can keep the cell homeostasis going, *in vitro* this cannot simply happen. Nevertheless, *in vivo* knockout brings also its own sets of challenges (e.g. disruption of different physiological cell functions due to off-target effects). Therefore, it is crucial to plan the knocking out of CD36 while keeping in mind the aforementioned possible challenges, above all if the aim of the study is to study such a target for possible novel atherosclerosis treatments.

CONCLUSION

The results gathered on CRISPR/Cas9, prime editing, and programmed RNA-editing, although scarce and not quantitatively assessable, suggest that programmed RNA-editing still has a long way to go before it has a more tangible and comparable potential as it is in the case for CRISPR/Cas9.

This thesis has shown how the lack of similar studies and data makes it quite challenging to determine objectively which tool actually holds the most promising qualities for use as clinical tools. Therefore, it is safe to assume that all of them have equal potential in translational medicine.

In the future, more data on the matter should be gathered and analysed in order to give a more quantitative perspective on the real potential and future perspectives of each tool here examined. Yet, even by just having a glimpse of them, it has been possible herein to take at least a snap-shot of the current state-of-the-art of biomedical engineering editing tools, which ultimately help us to come to some conclusions.

One of these conclusions is that each tool holds the highest potential in translational medicine, if they each were used for different specific diseases. Indeed, from the results gathered it is possible to speculate that CRISPR/Cas9 double strand editing has great potentiality for those diseases in which an *ex-vivo* treatment is applicable (e.g. heritable blood disorders such as sickle disease), and for diseases affecting immune privileged tissues and organs. Whilst the prime editing, although it is also a CRISPR/Cas9-based tool, appears to be more promising for treatment of carcinoma and heritable human monogenic diseases. Additionally, both methods could hold great potential for *in vivo* treatment if paired with a nanoparticle delivery system, which is an aspect that should be further explored in future research. Finally, programmed RNA-editing appears to be most promising for those diseases in which the production of a defective protein is due to a monogenic disease, where there are point mutations that can be edited post-transcriptionally on the mRNA without disrupting the actual gene. This last tool in particular should receive more attention, and more research should be done on the development and optimization of research methods that use this tool. This is important given the fact that opposite to CRISPR, possible off-target effects would not create permanent damage thanks to the non-permanent nature of mRNA. Therefore, since all three tools mentioned herein seem to have all great potential in transcriptional medicine, they should not be seen as a better or worse alternative to each other, but rather different solutions for different diseases.

REFERENCES

Abudayyeh O. O., Gootenberg J. S., Konermann S., Joung J., Slaymaker I. M., Cox D. B., Shmakov S., Makarova K. S., Semenova E., Minakhin L., Severinov K., Regev A., Lander E. S., Koonin E. V., Zhang F. (2016) C2c2 is a single-component programmable RNA-guided RNA-targeting CRISPR effector. *Science*, 353(6299): aaf5573.

Aida T., Wilde J. J., Yang L., Hou Y., Li M., Xu D., Lin J., Qi P., Lu Z., Feng G. (2021) Prime editing primarily induces undesired outcome in mice. *bioRxiv:* 10.1101/2020.08.06.239723 (August 6, 2020).

Aisling M. F. & Andrew R. G. (2018) Adenosine deaminase deficiency: A review. *Orphanet Journal of Rare Diseases,* 13(65).

Akinc A., Querbes W., De S., Qin J., Frank-Kamenetsky M., Narayanannair Jayaprakash K., Jayaraman M., Rajeev K. G., Cantley W. L., Dorkin J. R., Butler J. S., Qin L., Racie T., Sprague A., Fava E., Zeigerer A., Hope M. J., Zerial M., Sah D. W. Y., Fitzgerald K., Tracy M. A., Manoharan M., Koteliansky V., de Fougerolles A., Maier M. A. (2010) Targeted Delivery of RNAi Therapeutics With Endogenous and Exogenous Ligand-Based Mechanisms. *Molecular Therapy,* 18: 1357-1364.

Alessio M., De Monte L., Scirea A., Gruarin P., Tandon N. N., and Sitia R. (1996) Synthesis, Processing, and Intracellular Transport of CD36 during Monocytic Differentiation. *The Journal of Biological Chemistry,* 271(3): 1770-1775.

Álvarez‐Arellano L., Pedraza‐Escalona M., Blanco‐Ayala T., Camacho‐Concha N., Cortés‐Mendoza J., Pérez‐Martínez L., Pedraza‐Alva G. (2017) Autophagy impairment by caspase‐1‐dependent inflammation mediates memory loss in response to beta‐Amyloid peptide accumulation. *Journal of Neuroscience Research*, 96(2):234-246.

Anzalone A. V., Randolph P. B., Davis J. R., Sousa A. A., Koblan L. W., Levy J. M., Chen P. J., Wilson C., Newby G. A., Raguram A., Liu D. R. (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature*, 576:149-157.

Anzalone A.V., Randolph P. B., Davis J. R., Sousa A. A., Koblan L. W., Levy J. M., Chen P. J., Wilson C., Newby G. A., Raguram A. & Liu D. R. (2019) Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature,* 576: 149–157.

Armesilla A. L. and Vega M. A. (1994) Structural organization of the gene of human CD36 glycoprotein. *The Journal of Biological Chemistry*, 269(29):18985-18991.

Azad M. T. A., Bhakta S. & Tsukahara T. (2017) Site-directed RNA editing by adenosine deaminase acting on RNA (ADAR1) for correction of the genetic code in gene therapy. *Gene Therapy,* 24(12): 779–786.

Bawa K.K., Krance S.H., Herrmann N., Cogo-Moreira H., Ouk M., Yu D., Wu C., Black S. E., Lanctôt K. L., Swardfager W. (2020) A peripheral neutrophil-related inflammatory factor predicts a decline in executive function in mild Alzheimer's disease. *Journal of Neuroinflammation*, 17: 84.

Bentzon J. F., Otsuka F., Virmani R., and Falk E. (2014) Mechanisms of plaque formation and rupture. Circulation Research, 114:1852-1866.

Bhakta S., Azad M. T. A. & Tsukahara T. (2018) Genetic code restoration by artificial RNA editing of Ochre stop codon with ADAR1 deaminase. *Protein Engineering, Design and Selection,* 31(12): 1–8.

Birkner K., Loos J., Gollan R., Steffen F., Wasser B., Ruck T., Meuth S. G., Zipp F., & Bittner S. (2019) Neuronal ICAM-5 Plays a Neuroprotective Role in Progressive Neurodegeneration. *Frontiers in Neurology*, 10:205.

Bosch J. A., Birchak G. and Perrimon N. (2021) Precise genome engineering in *Drosophila* using prime editing. *Proceedings of the National Academy of Sciences of the United States of America,* 118(1): e2021996118.

Bosshart H, Heinzelmann M. (2016) THP-1 cells as a model for human monocytes. *Annals of Translational Medicine,* 4(21): 438.

Brachène A. C., Castela A., Op de Beeck A., Mirmira R. G., Marselli L., Marchetti P., Masse C., Miao W., Leit S., Evans-Molina C., Eizirik D. L. (2020) Preclinical evaluation of tyrosine

kinase 2 inhibitors for human beta-cell protection in type 1 diabetes. *Diabetes, Obesity and Metabolism*, 22(10):1827.1836.

Brennicke A., Marchfelder A., Binder S. (1999) RNA editing. *FEMS Microbiology Reviews,* 23(3): 297-316.

Buttorff C., Ruder T., Bauman M. (2017) Multiple Chronic Conditions in the United States. Santa Monica, CA: Rand Corp. retrieved from: https://www.rand.org/content/dam/rand/pubs/tools/TL200/TL221/RAND_TL221.pdf accessed on the 1st of March 2021.

CDC.gov "Health and Economic Costs of Chronic Diseases" retrieved from https://www.cdc.gov/chronicdisease/about/costs/index.htm#ref1 accessed on the 1st of March 2021.

Celarain N. and Tomas-Roig J. (2020) Aberrant DNA methylation profile exacerbates inflammation and neurodegeneration in multiple sclerosis patients. *Journal of Neuroinflammation*, 17(1):21.

Chen D., Tang J., Li B., Hou L., Wang X. & Kang L. (2018) CRISPR/Cas9-mediated genome editing induces exon skipping by complete or stochastic altering splicing in the migratory locust. *BMC Biotechnology*, (18):60.

Cheng D., Iqbal J., Devenny J., Chu C. H., Chen L., Dong J., Seethala R., Keim W. J., Azzara A. V., Lawrence R. M., Pelleymounter M. A., Hussain M. M. (2008) Acylation of acylglycerols by acyl coenzyme A:diacylglycerol acyltransferase 1 (DGAT1). Functional importance of DGAT1 in the intestinal fat absorption. *Journal of Biological Chemistry*, 283(44): 29802-11.

Chen Z., Liu F., Chen Y., Liu J., Wang X., Chen A. T., Deng G., Zhang H., Liu J., Hong Z., Zhou J. (2017) Targeted Delivery of CRISPR/Cas9‐Mediated Cancer Gene Therapy via Liposome‐Templated Hydrogel Nanoparticles. *Advanced Functional Materials,* 27(46): e1703036.

Chistiakov D. A., Bobryshev Y. V., Orekhov A. N. (2016) Macrophage-mediated cholesterol handling in atherosclerosis. *Journal of Cell Biology*, 20(1):17-28.

Choi J., Lee H. W., Suk K. (2011) Increased plasma levels of lipocalin 2 in mild cognitive impairment. *Journal of Neurological Sciences*, 305(1-2):28-33.

CHOPCHOP "Instructions" retrieved from: https://chopchop.cbu.uib.no/instructions accessed on the 9th of October 2020.

Christofi T. and Zaravinos A. (2019) RNA editing in the forefront of epitranscriptomics and human health. *Journal of Translational Medicine,* 17: 319.

Cohrs R. J., Martin T., Ghahramani P., Bidaut L., Higgins P. J., Shahzad A. (2015) Translational Medicine definition by the European Society for Translational Medicine. *New Horizons in Translational Medicine,* 2(3): 86-88.

Cong L., Ran F.A., Cox D., Lin S., Barretto R., Habib N., Hsu P.D., Wu X., Jiang W., Marraffini L.A., et al. (2013) Multiplex genome engineering using CRISPR/Cas systems. *Science*, 339:819-823.

Cox D. B. T., Gootenberg J. S., Abudayyeh O. O., Franklin B., Kellner M. J., Joung J., Zhang F. (2017) RNA editing with CRISPR-Cas13. *Science*, 358(6366): 1019-1027.

"CRISPR fly design, gRNA IVT: Production of gRNAs by in-vitro transcription (IVT)", retrieved from: http://www.crisprflydesign.org/grnatranscription/ accessed on the 23rd October 2020

Cruz Hernández J. C., Bracko O., Kersbergen C. J., Muse V., Haft-Javaherian M., Berg M., Park L., Vinarcsik L. K., Ivasyk I., Rivera D. A., Kang Y., Cortes-Canteli M., Peyrounette M., Doyeux V., Smith A., Zhou J., Otte G., Beverly J. D., Davenport E., Davit Y., Schaffer C. B. (2019). Neutrophil adhesion in brain capillaries reduces cortical blood flow and impairs memory function in Alzheimer's disease mouse models. *Nature Neuroscience*, 22(3):413–420.

David T., Ling S. F., and Barton A. (2018) Genetic of immune -mediated inflammatory diseases. *Clinical and Experimental Immunology,* 193: 3-12.

Daviet L. and McGregor J. L. (1997) Vascular biology of CD36: roles of this new adhesion molecule family in different disease states. *Thrombosis and Haemostasis,* 78 (1): 65–69.

Debacker A. J., Voutila J., Catley M., Blakey D., Habib N. (2020) Delivery of Oligonucleotides to the Liver with GalNAc: From Research to Registered Therapeutic Drug. *Molecular Therapy,* 28(8): 1759-1771.

Dekens D. W., Naudé P. J. W., Engelborghs S., Vermeiren Y., Van Dam D., Oude Voshaar R. C., Eisel U. L. M., De Deyn P. P. (2017) Neutrophil Gelatinase-Associated Lipocalin and Its Receptors in Alzheimer's Disease (AD) Brain Regions: Differential Findings in AD with and Without Depression. *Journal of Alzheimer's Disease*, 55(2):763-776.

Doench J.G., Fusi N., Sullender M., et al. (2016) Optimized sgRNA design to maximize activity and minimize off-target effects of CRISPR/Cas9. *Nature Biotechnology*, 34(2):184-191.

Dong Y., Legarde J., Xicota L., Corne H., Chantran Y., Chaigneau T., Crestani B., Bottlaender M., Potier M., Aucouturier P., Dorothée G., Sarazin M., Elbim C. (2018) Neutrophil hyperactivation correlates with Alzheimer's disease progression. *Annals of Neurology*, 83(2):387-405.

Doudna J. A. and Charpentier E. (2014) The new frontier of genome engineering with CRISPR/Cas9. *Science*, 346(6213):1077.

Eggington J. M., Greene T., Bass B. L. (2011) Predicting sites of ADAR editing in double-stranded RNA. *Nature Communications,* 2: 319.

Eizirik D. L. and Colli M. L. (2020) Revisiting the role of inflammation in the loss of pancreatic beta-cells in T1DM. *Nature Reviews Endocrinology*, 16:611-612.

Engelhardt B., Wolburg-Buchholz K., Wolburg H. (2001) Involvement of the choroid plexus in central nervous system inflammation. *Microscopy Research and Technique,* 52:112-129.

Erdman L. K., Cosio G., Helmers A. J., Gowda D. C., Grinstein S., Kain K. C. (2009) CD36 and TLR interactions in inflammation and phagocytosis: implications for malaria. *Journal of Immunology,* 183(10): 6452–6459.

Esrick E. B., Lehmann L. E., Biffi A., Achebe M., Brendel C., Ciuculescu M. F., Daley H., MacKinnon B., Morris E., Federico A., Abriss D., Boardman K., Khelladi R., Shaw K., Negre H., Negre O., Nikiforow S., Ritz J., Pai S. Y., London W. B., Dansereau C., Heeney M. M.,

Armant M., Manis J. P., Williams D. A. (2020) Post-transcriptional genetic silencing of BCL11A to treat sickle cell disease. *The New England Journal of Medicine*, retrieved from: www.nejm.org/doi/10.1056/NEJMoa2029392 accessed on the 17th of December 2020.

Esrick E. B., Lehmann L. E., Biffi A., Achebe M., Brendel C., Ciuculescu M. F., Daley H., MacKinnon B., Morris E., Federico A., Abriss D., Boardman K., Khelladi R., Shaw K., Negre H., Negre O., Nikiforow S., Ritz J., Pai S. Y., London W. B., Dansereau C., Heeney M. M., Armant M., Manis J. P., Williams D. A. (2021) Post-Transcriptional genetic silencing of *BCL11A* to treat sickle cell disease. *The New England Journal of Medicine,* 384(3):205-215.

Feehan K. T. and Gilroy D. W. (2019) Is resolution the end of inflammation? Trends in Molecular Medicine. 25(3):198-214.

Ferguson L. R. (2010) Chronic inflammation and mutagenesis. *Mutation Research - Fundamentals and Molecular mechanisms of mutagenesis*, 690:3-11.

Ferrucci L., & Fabbri E. (2018). Inflammaging: chronic inflammation in ageing, cardiovascular disease, and frailty. *Nature reviews - Cardiology*, 15(9): 505–522.

Fine N., Chadwick J. W., Sun C., Parbhakar K. K., Khoury N., Barbour A., Goldberg M., Tenenbaum H. C., Glogauer M. (2020) Periodontal inflammation primes the systemic innate immune response. *Journal of Dental Research*, retrieved from: https://doi.org/10.1177/0022034520963710 accessed on the 6th of January 2021.

Finn J. D., Smith A. R., Patel M. C., Shaw L., Youniss M. R., van Heteren J., Dirstine T., Ciullo C., Lescarbeau R., Seitzer J., Shah R. R., Shah A., Ling D, Growe J., Pink M., Rohde E., Wood K. M., Salomon W. E., Harrington W. F., Dombrowski C., Strapps W. R., Chang Y., Morrissey D. V. (2018) A Single Administration of CRISPR/Cas9 Lipid Nanoparticles Achieves Robust and Persistent In Vivo Genome Editing. *Cell Reports,* 22(9): 2227-2235.

"First CRISPR therapy dosed" (2020) *Nature Biotechnology,* 38, 382, retrieved from: https://doi.org/10.1038/s41587-020-0493-4 accessed on the 15th of February 2021.

Fonseca D. M., Hand T. W., Han S. J., Gerner M. Y., Glatman Zaretsky A., Byrd A. L., Harrison O. J., Ortiz A. M., Quinones M., Trinchieri G., Brenchley J. M., Brodsky I. E., Germain R. N., Randolph G. J., & Belkaid Y. (2015) Microbiota-Dependent Sequelae of Acute Infection Compromise Tissue-Specific Immunity. *Cell,* 163(2):354–366.

Franceschi C., Bonafè M., Valensin S., Olivieri F., De Luca M., Ottaviani E., De Benedictis G. (2000) Inflamm-aging. An evolutionary perspective on immunosenescence. *Annals of the New York Academy of Sciences*, 908:244-54.

Frangoul H., Altshuler D., Cappellini M.D., Chen Y.-S., Domm J., Eustace B.K., Foell J., de la Fuente J., Grupp S., Handgretinger R., Ho T.W., Kattamis A., Kernytsky A., Lekstrom-Himes J., Li A.M., Locatelli F., Mapara M.Y., de Montalembert M., Rondelli D., Sharma A., Sheth S., Soni S., Steinberg M.H., Wall D., Yen A., and Corbacioglu S. (2020) CRISPR/Cas9 gene editing for Sickle Cell Disease and β-Thalassemia. *The New England Journal of Medicine,* retrieved from: www.nejm.org/doi/full/10.1056/NEJMoa2031054 accessed on the 12th February 2021.

Frangoul H., Altshuler D., Cappellini M.D., Chen Y.-S., Domm J., Eustace B.K., Foell J., de la Fuente J., Grupp S., Handgretinger R., Ho T.W., Kattamis A., Kernytsky A., Lekstrom-Himes J., Li A.M., Locatelli F., Mapara M.Y., de Montalembert M., Rondelli D., Sharma A., Sheth S., Soni S., Steinberg M.H., Wall D., Yen A., and Corbacioglu S. (2020) CRISPR/Cas9 gene editing for Sickle Cell Disease and β-Thalassemia. *The New England Journal of Medicine*, retrieved from: www.nejm.org/doi/full/10.1056/NEJMoa2031054 accessed on the 17th of December 2020.

Fries J. F., Williams C. A., Bloch D. A., Michel B. A. (1991) Nonsteroidal anti-inflammatory drug-associated gastropathy: incidence and risk factor models. *The Medical Journal of Medicine*, 91(3):213–22.

Fry L. E., Peddle C. F., Barnard A. R., McClements M. E. and MacLaren R. E.(2020) RNA editing as therapeutic approach for retinal gene therapy requiring long coding sequences. *International Journal of Molecular Sciences*, 21(3):777.

Furman D., Campisi J., Verdin E., Carrera-Bastos P., Targ S., Franceschini C., Ferrucci L., Gilroy D. W., Fasano A., Miller G. W., Miller A. H., Mantovani A., Weyand C. M., Barzilai N., Goronzy J. J., Rando T. A., Effros B. R., Lucia A., Kleinstreuer N., Slavich G. M. (2019) Chronic inflammation in the etiology of disease across the lifespan. *Nature Medicine*, 25(12):1822-1832.

Galkina E. and Ley K. (2009) Immune and inflammatory mechanisms of atherosclerosis. *Annual Review of Immunology*, 27:165-167.

Gootenberg J. S., Abudayyeh O. O., Kellner M. J., Joung J., Collins J. J., Zhang F. (2018) Multiplexed and portable nucleic acid detection platform with Cas13, Cas12a, and Csm6. *Science,* 360(6387): 439-444.

Gootenberg J. S., Abudayyeh O. O., Lee J. W., Essletzbichler P., Dy A. J., Joung J., Verdine V., Donghia N., Daringer N. M., Freije C. A., Myhrvold C., Bhattacharyya R. P., Livny J., Regev A., Koonin E. V., Hung D. T., Sabeti P. C., Collins J. J., Zhang F. (2017) Nucleic acid detection with CRISPR-Cas13a/C2c2. *Science,* 356: 438-442.

Gregersen P. K., Silver J., Winchester R. J. (1987) The shared epitope hypothesis an approach to understanding the molecular genetics of susceptibility to rheumatoid arthritis. *Arthritis and rheumatology,* 30(11):1205-1213.

Grivennikov S. I., Greten F. R., and M. Karin (2010) Immunity, Inflammation, and Cancer. *Cell,* 140(6):883-99.

Gupta V., Bhavanasi S., Quadir M., Singh K., Ghosh G., Vasamreddy K., Ghosh A., Siahaan T. J., Banerjee S., & Banerjee S. K. (2019) Protein PEGylation for cancer therapy: bench to bedside. *Journal of cell communication and signaling*, 13(3): 319–330.

Hall J. R., Wiechmann A. R., Johnson L. A., Edwards M., Barber R. C., Winter A. S., Singh M., & O'Bryant S. E. (2013) Biomarkers of vascular risk, systemic inflammation, and microvascular pathology and neuropsychiatric symptoms in Alzheimer's disease. *Journal of Alzheimer's disease*, 35(2):363–371.

Hanna R. E. and Doench J. G. (2020) Design and analysis of CRISPR-Cas experiments. *Nature Biotechnology,* (38):813-823.

Hirschhorn R., Tzall S., Ellenbogen A. & Orkin S. H. (1989) Identification of a point mutation resulting in a heat-labile adenosine deaminase (ADA) in two unrelated children with partial ADA deficiency. *Journal of Clinical Investigation,* 83(2): 497–501.

Holmes C., Cunningham C., Zotova E., Culliford D., & Perry V. H. (2011) Proinflammatory cytokines, sickness behavior, and Alzheimer disease. *Neurology*, 77(3): 212–218.

Howard R. L., Avery A. J., Slavenburg S., Royal S., Pipe G., Lucassen P., Pirmohamed M. (2007) Which drugs cause preventable admissions to hospital? A systematic review. *British Journal of Clinical Pharmacology,* 63(2):136–47.

Hsu P.D., Scott D.A., Weinstein J.A., Ran F.A., Konermann S., Agarwala V., Li Y., Fine E.J., Wu X., Shalem O., Cradick T. J., Marraffini L. A., Bao G., Zhang F. (2013) DNA targeting specificity of RNA-guided Cas9 nucleases. *Nature Biotechnology*, 31:827-832.

Huizinga T. W., Amos C. I., van der Helm-van Mil A. H., Chen W., van Gaalen F. A., Jawaheer D., Schreuder G. M., Wener M., Breedveld F. C., Ahmad N., Lum R. F., de Vries R. R., Gregersen P. K., Toes R. E., Criswell L. A. (2005) Refining the complex rheumatoid arthritis phenotype based on specificity of the HLA-DRB1 shared epitope for antibodies to citrullinated proteins. *Arthritis and rheumatology*, 52(11):3433-8.

Jackson D. A., Symons R. H. and Berg P. (1972) Biochemical method for inserting new genetic information into DNA of Simian Virus 40: circular SV40 DNA molecules containing lambda phage genes and the galactose operon of Escherichia coli. *Proceedings of the National Academy of Science of the United States of America*, 69(10):2904-2909.

Jinek M., Chylinski K., Fonfara I., Hauer M., Doudna J. A., Charpentier E. (2012) A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science,* 337(6096):816-821.

Jiskoot W., Randolph T. W., Volkin D. B., Middaugh C. R., Schöneich C., Winter G., Friess W., Crommelin D. J., Carpenter J. F. (2012) Protein instability and immunogenicity: roadblocks to clinical application of injectable protein delivery systems for sustained release. *Journal of Pharmaceutical Sciences,* 101(3): 946-54.

Jubair L., Lam A. K., Fallaha S., McMillan N. A. J. (2021) CRISPR/Cas9-loaded stealth liposomes effectively cleared established HPV16-driven tumours in syngeneic mice. *PLoS ONE,* 16(1): e0223288.

Kantor A., McClements M. E., MacLaren R. E. (2020) CRISPR/Cas9 DNA Base-Editing and Prime-Editing. *International Journal of Molecular Sciences,* 21(17): 6240.

Katrekar D., Chen G., Meluzzi D., Ganesh A., Worlikar A., Shih Y. R., Varghese S., Mali P. (2019) In vivo RNA editing of point mutations via RNA-guided adenosine deaminases. *Nature Methods,* 16(3):239-242.

Katrekar D., Yen J., Xiang Y., Saha A., Meluzzi D., Savva Y., Mali P. (2021) Robust RNA editing via recruitment of endogenous ADARs using circular guide RNAs. *bioRxiv:* https://doi.org/10.1101/2021.01.12.426286 (January 13, 2021).

Kim H. K., Yu G., Park J., Min S., Lee S., Yoon S. & Kim H. H. (2020) Predicting the efficiency of prime editing guide RNAs in human cells. *Nature Biotechnology,* 39: 198-206.

Konermann S., Lotfy P., Brideau N. J., Oki J., Shokhirev M. N., Hsu P. D. (2018) Transcriptome Engineering with RNA-Targeting Type VI-D CRISPR Effectors. *Cell,* 173(3):665-676.e14.

Kulakova O. G., Kabilov M. R., Danilova L. V., Popova E. V., Baturina O. A., Tsareva E. Y., Baulina N. M., Kiselev I. S., Boyko A. N., Favorov A. V., Favorova O. O., Vlassov V. V. (2016) Whole-Genome DNA Methylation Analysis of Peripheral Blood Mononuclear Cells in Multiple Sclerosis Patients with Different Disease Courses. *Acta Naturae,* 8(3):103-110.

Lai K. S. P., Liu C. S., Rau A., Lanctôt K. L., Köhler C. A., Pakosh M., Carvalho A. F., Herrmann N. (2017) Peripheral inflammatory markers in Alzheimer's disease: a systematic review and meta-analysis of 175 studies. *Journal of Neurology, Neurosurgery and Psychiatry,* 88(10):876-882.

Lane D. A., Kunz G., Olds R. J. & Thein S. L. (1996) Molecular genetics of antithrombin deficiency. *Blood Reviews,* 10(2): 59–74.

Lehmann K. A. and Bass B. L. (2000) Double-stranded RNA adenosine deaminases ADAR1 and ADAR2 have overlapping specificities. *Biochemistry*, 39(42): 12875–12884.

Lendford H. (2020) CRISPR gene therapy shows promise against blood diseases. *Nature,* 588:383.

Lin P., Qin S., Pu Q., Wang Z., Wu Q., Gao P., Schettler J., Guo K., Li R., Li G., Huang C., Wei Y., Gao G. F., Jiang J., Wu M. (2020) CRISPR-Cas13 Inhibitors Block RNA Editing in Bacteria and Mammalian Cells. *Molecular Cell*, 78(5):850-861.e5.

Lonkar P. and Dedon P. C. (2010) Reactive species and DNA damage in chronic inflammation: reconciling chemical mechanisms and biological fates. *International Journal of Cancer*, 128(9): 1999-2009.

MacDonald B. T., Tamai K., & He X. (2009) Wnt/beta-catenin signaling: components, mechanisms, and diseases. *Developmental Cell*, 17(1): 9–26.

Magini D., Giovani C., Mangiavacchi S., Maccari S., Cecchi R., Ulmer J. B., De Gregorio E., Geall A. J., Brazzoli M., Bertholet S. (2016) Self-amplifying mRNA vaccines expressing multiple conserved influenza antigens confer protection against homologous and heterosubtypic viral challenge. *PLoS ONE,* 11: e0161193.

Making changes to the molecular messengers that create proteins might offer flexible therapies for cancer, pain or high cholesterol, in addition to genetic disorders. *Nature,* 578: 24-27.

Manils J., Webb L. V., Howes A., Janzen J., Boeing S., Bowcock A. M., Ley S. C. (2020) CARD14E138A signalling in keratinocytes induces TNF-dependent skin and systemic inflammation. *Elife,* 9:e56720.

Martin A. B., Hartman M., Lassman D., Catlin A. (2020) National Health Care Spending In 2019: Steady Growth For The Fourth Consecutive year. *Health Affair,* 40(1): 1-11.

Mellett, M. (2020) Regulation and dysregulation of CARD14 signalling and its physiological consequences in inflammatory skin disease. *Cellular Immunology,* 354:104147.

Mellett M., Meier B., Mohanan D., Schairer R., Cheng P., Satoh T. K., Kiefer B., Ospelt C., Nobbe S., Thome M., Contassot E., French L. E. (2018) CARD14 Gain-of-Function Mutation Alone Is Sufficient to Drive IL-23/IL-17-Mediated Psoriasiform Skin Inflammation In Vivo. *Journal of Investigative Dermatology,* 138(9):2010-2023.

Merkle T., Merz S., Reautschnig P., Blaha A., Li Q., Vogel P., Wettengel J., Li J. B., Stafforst T. (2019) Precise RNA editing by recruiting endogenous ADARs with antisense oligonucleotides. *Nature Biotechnology,* 37(2):133-138.

Michlits G., Jude J., Hinterndorfer M., de Almeida M., Vainorius G., Hubmann M., Neumann T., Schleiffer A., Burkard T. R., Fellner M., Gijsbertsen M., Traunbauer A., Zuber J. (2020) Multilayered VBC score predicts sgRNAs that efficiently generate loss-of-function alleles. *Nature Methods*, (17):708–716.

Milla P., Dosio F., Cattle L. (2012) PEGylation of proteins and liposomes: a powerful and flexible strategy to improve drug delivery. *Current Drug Metabolism,* 13(1):105-119.

Milone C. M. & O' Doherty (2018) Clinical use of lentiviral vectors. *Leukemia,* 32: 1529-1541.

Monaco S., Nicholas R., Reynolds R., Migliozzi R. (2020) Intrathecal inflammation in progressive multiple sclerosis. *International Journal of Molecular Sciences,* 21(21):8217.

Montague T. G., Cruz J. M., Gagnon J. A., Church G. M., Valen E. (2014) CHOPCHOP: a CRISPR/Cas9 and TALEN web tool for genome editing. *Nucleic Acids Research,* 42(W1):W401–W407.

Montiel-González M. F., Vallecillo-Viejo I. C., & Rosenthal J. J. (2016) An efficient system for selectively altering genetic information within mRNAs. *Nucleic acids research*, 44(21): e157.

Myhrvold C., Freije C. A., Gootenberg J. S., Abudayyeh O. O., Metsky H. C., Durbin A. F., Kellner M. J., Tan A. L., Paul L. M., Parham L. A., Garcia K. F., Barnes K. G., Chak B., Mondini A., Nogueira M. L., Isern S., Michael S. F., Lorenzana I., Yozwiak N. L., MacInnis B. L., Bosch I., Gehrke L., Zhang F., Sabeti P. C. (2018) Field-deployable viral diagnostics using CRISPR-Cas13. *Science,* 360(6387): 444-448.

Naert T., Tulkens D., Edwards N. A., Carron M., Shaidani N., Wlizla M., Boel A., Demuynck S., Horb M. E., Coucke P., Willaert A., Zorn A. M. & Vleminckx K. (2020) Maximizing CRISPR/Cas9 phenotype penetrance applying predictive modeling of editing outcomes in Xenopus and zebrafish embryos. *Scientific Reports,* (10):14662.

Nasef A. N., Mehta S., Ferguson L. R. (2017) Susceptibility to chronic inflammation: an update. Archive of Toxicology, 91:1131-1141.

Naudé P. J. W., Nyakas C., Eiden L. E., Ait-Ali D., van der Heide R., Engelborghs S., Luiten P. G., De Deyn P. P., den Boer J. A. & Eisel U. L. (2012) Lipocalin 2: novel component of proinflammatory signaling in Alzheimer's disease. *FASEB journal : official publication of the Federation of American Societies for Experimental Biology,* 26(7):2811–2823.

Netea M. G., Balkwill F., Chonchol M., Cominelli F., Donath M. Y., Giamarellos-Bourboulis E. J., Golenbock D., Gresnigt M. S., Heneka M. T., Hoffman H. M., Hotchkiss R., Joosten L. A. B., Kastner D. L., Korte M., Latz E., Libby P., Mandrup-Poulsen T., Mantovani A., Mills K. H. G., Nowak K. L., O'Neill L. A., Pickkers P., van der Poll T., Ridker P. M., Schalkwijk J., Schwartz D. A., Siegmund B., Steer C. J., Tilg H., van der Meer J. W. M., van de Veerdonk F. L., Dinarello C. A. (2017) A guiding map for inflammation. *Nature Immunology,* 18:826-831.

Olds R. J., Lane D. A., Ireland H., Finazzi G., Barbui T., Abildgaard U., Girolami A., Thein S. L. (1991) A common point mutation producing type 1A antithrombin III deficiency: AT129 CGA to TGA (Arg to Stop). *Thrombosis Research*, 64(5): 621–625.

Op De Beeck A. and Eizirik D. L. (2016) Viral infections in type 1 diabetes mellitus - why the beta-cells?. *Nature Reviews Endocrinology*, 12:263-273.

Paetau S., Rolova T., Ning L., Gahmberg C. G. (2017) Neuronal ICAM-5 inhibits microglia adhesion and phagocytosis and promotes an anti-inflammatory response in LPS stimulated microglia. *Frontiers in Molecular Neuroscience,* 10:1–12.

Park Y. M. (2014) CD36, a scavenger receptor implicated in atherosclerosis. *Experimental & Molecular Medicine,* 46, e99.

Phillips C. M., Chen L. W., Heude B., Bernard J. Y., Harvey N. C., Duijts L., Mensink-Bout S. M., Polanska K., Mancano G., Suderman M., Shivappa N., & Hébert J. R. (2019) Dietary Inflammatory Index and Non-Communicable Disease Risk: A Narrative Review. *Nutrients*, 11(8): 1873.

Pietronigro E. C., Della Bianca V., Zenaro E., & Constantin G. (2017) NETosis in Alzheimer's Disease. *Frontiers in Immunology*, 8: 211.

Polson A. G. and Bass B. L. (1994) Preferential selection of adenosines for modification by double-stranded RNA adenosine deaminase. *EMBO Journal,* 13(23): 5701–5711.

Primer-BLAST: https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi

Qu L., Yi Z., Zhu S., Wang C., Cao Z., Zhou Z., Yuan P., Yu Y., Tian F., Liu Z., Bao Y., Zhao Y., Wei W. (2019) Programmable RNA editing by recruiting endogenous ADAR using engineered RNAs. *Nature Biotechnology,* 37: 1059–1069.

Ramírez-Moya J., Baker A. R., Slack F. J. & Santisteban P. (2020) ADAR1-mediated RNA editing is a novel oncogenic process in thyroid cancer and regulates miR-200 activity. *Oncogene,* 39: 3738–3753.

Reardon S. (2020) "Step aside CRISPR, RNA editing is taking off" retrieved from: https://www.nature.com/articles/d41586-020-00272-5 accessed on the 30th of March 2021.

Richner J. M., Himansu S., Dowd K. A., Butler S. L., Salazar V., Fox J. M., Julander J. G., Tang W. W., Shresta S., Pierson T. C., Ciaramella G., Diamond M. S. (2017) Modified mRNA vaccine protects against Zika virus infection. *Cell,* 168(6): 1114-1125.e10.

Ridker P. M., Hennekens C. H., Buring J. E., Rifai N. (2000) C-reactive protein and other markers of inflammation in the prediction of cardiovascular disease in women. *New England Journal of Medicine,* 342:836-43.

Ridker P. M. (2003) Clinical application of C-reactive protein for cardiovascular disease detection and prevention. *Circulation,* 107:363-9.

Roh J. S., & Sohn D. H. (2018) Damage-Associated Molecular Patterns in Inflammatory Diseases. *Immune network*, 18(4), e27.

Rosenblum D., Gutkin A., Dammes N., Peer D. (2020) Progress and challenges towards CRISPR/Cas clinical translation. *Advanced Drug Reviews,* 154-155:176-186

Sakurai H., Kawabata K., Sakurai F., Nakagawa S., Mizuguchi H. (2007) Innate immune response induced by gene delivery vectors. *International Journal of Pharmaceutics,* 354(1-2): 9-15.

Sanjana N.E., Shalem O., Zhang F. (2014) Improved lentiviral vectors and genome-wide libraries for CRISPR screening. *Nature Methods,* 11(8): 783-784.

Schene I. F., Joore I. P., Oka R., Mokry M., van Vugt A. H., van Boxtel R., van der Doef H. P., van der Laan L. J., Verstegen M. M., van Hasselt P. M., Nieuwenhuis E. E., Fuchs S. A.

(2020) Prime editing for functional repair in patient-derived disease models. *Nature Communications*, 11:5352.

Schene I. F. , Joore I. P. , Oka R., Mokry M., van Vugt A. H. M., van Boxtel R., van der Doef H. P. J., van der Laan L. J. W., Verstegen M. M. A., van Hasselt P. M., Nieuwenhuis E. E. S. & Fuchs S. A. (2020) Prime editing for functional repair in patient-derived disease models. *Nature Communications,* 11: 5352.

Shalem O., Sanjana N.E., Hartenian E., Shi X., Scott D.A., Mikkelsen T., Heckl D., Ebert B. L., Root D. E., Doench J. G., Zhang F. (2014) Genome-scale CRISPR/Cas9 knockout screening in human cells. *Science,* 343(6166): 83-87.

Sheridan C. (2021) First small-molecule drug targeting RNA gains momentum. *Nature Biotechnology,* 39: 6-8.

Shigekawa K. and Dower W. J. (1988) Electroporation of eukaryotes and prokaryotes: a general approach to the introduction of macromolecules into cells. *Biotechniques*, 6(8):742-51.

Shilo B. Z. and Schejter E. D. (2011) Regulation of developmental intercellular signalling by intracellular trafficking. *The EMBO journal*, 30(17): 3516–3526.

Sigma: https://www.sigmaaldrich.com/norway.html

Song X., Liu C., Wang N., Huang H., He S., Gong C., Wei Y. (2021) Delivery of CRISPR/Cas systems for cancer gene therapy and immunotherapy. *Advanced Drug Delivery Reviews,* 168: 158-180.

Stafforst T. and Schneider M. F. (2012) An RNA-deaminase conjugate selectively repairs point mutations. *Angewandte Chemie International Edition,* 51: 11166-11169.

Stafforst T. and Schneider M. F. (2012) An RNA-deaminase conjugate selectively repairs point mutations. *Angewandte Chemie International Edition,* 51(44):11166-11169.

Sundberg J. P., Pratt C. H., Silva K. A., Kennedy V. E., Qin W., Stearns T. M., Frost J., Sundberg B. A., Bowcock A. M. (2019) Gain of function p.E138A alteration in Card14 leads to psoriasiform skin inflammation and implicates genetic modifiers in disease severity. *Experimental and Molecular Pathology,* 110:104286.

Surace A. E. A. and Hedrich C. M. (2019) The role of epigenetics in autoimmune/inflammatory disease. *Frontiers in Immunology,* 10:1525.

Swardfager W., Lanctôt K., Rothenburg L., Wong A., Cappell J., Herrmann N. (2010) A meta-analysis of cytokines in Alzheimer's disease. *Biological Psychiatry,* 68(10):930-941.

Tabas I., Garcia-Cardena G., Owens G.K. (2015) Recent insights into the cellular biology of atherosclerosis. *Journal of Cell Biology,* 209(1):13-22.

Teoh P. J., An O., Chung T. H., Chooi J. Y., Toh S. H. M., Fan S., Wang W., Koh B. T. H., Fullwood M. J., Ooi M. G., de Mel S., Soekojo C. Y., Chen L., Ng S. B., Yang H., Chng W. J. (2018) Aberrant hyperediting of the myeloma transcriptome by ADAR1 confers oncogenicity and is a marker of poor prognosis. *Blood,* 132: 1304–1317.

Terns M. P. (2018) CRISPR-Based Technologies: Impact of RNA-Targeting Systems. *Molecular Cell*, 72(3):404-412.

The Broad Institute Genetic Perturbation Platform, How to use the GPP sgRNA designer (CRISPRko), retrieved from: https://portals.broadinstitute.org/gpp/public/analysis-tools/sgrna-design-help accessed on the 9th of October 2020.

Thi E. P., Mire C. E., Ursic-Bedoya R., Geisbert J. B., Lee A. C. H., Agans K. N., Robbins M., Deer D. J., Fenton K. A., MacLachlan I., Geisbert T. W. (2014) Marburg virus infection in nonhuman primates: Therapeutic treatment by lipid-encapsulated siRNA. *Science Translational Medicine,* 6: 250ra116.

Thieman W. J. and Palladino M. A. (2014) Introduction to Biotechnology. *Pearson New International Edition*, 3rd Ed., pp.22.

ThermoFisher "Factor Influencing Transfection Efficiency" retrieved from: https://www.thermofisher.com/no/en/home/references/gibco-cell-culture-basics/transfection-basics/factors-influencing-transfection-efficiency.html accessed on the 7th of May 2021.

Thyme S. B., Akhmetova L., Montague T. G., Valen E. & Schier A. F. (2016) Internal guide RNA interactions interfere with Cas9-mediated cleavage. *Nature Communications*, 7:11750.

Tian K., Xu Y., Sahebkar A. & Xu S. (2020) CD36 in atherosclerosis: Pathophysiological mechanisms and therapeutic implications. *Current Atherosclerosis Reports,* 22(59).

Tsai S. Q., Zheng Z., Nguyen N. T., Liebers M., Topkar V. V., Thapar V., Wyvekens N., Khayter C., Iafrate A. J., Le L. P., Aryee M. J., and Joung J. K. (2015) GUIDE-Seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. *Nature Biotechnology,* 33(2):187-197.

Tsoi L. C., Spain S. L., Knight J., Ellinghaus E., Stuart P. E., Capon F., Ding J., Li Y., Tejasvi T., Gudjonsson J. E., Kang H. M., Allen M. H., McManus R., Novelli G., Samuelsson L., Schalkwijk J., Ståhle M., Burden A. D., Smith C. H., Cork M. J., Estivill X., Bowcock A. M., Krueger G. G., Weger W., Worthington J., Tazi-Ahnini R., Nestle F. O., Hayday A., Hoffmann P., Winkelmann J., Wijmenga C., Langford C., Edkins S., Andrews R., Blackburn H., Strange A., Band G., Pearson R. D., Vukcevic D., Spencer C. C., Deloukas P., Mrowietz U., Schreiber S., Weidinger S., Koks S., Kingo K., Esko T., Metspalu A., Lim H. W., Voorhees J. J., Weichenthal M., Wichmann H. E., Chandran V., Rosen C. F., Rahman P., Gladman D. D., Griffiths C. E., Reis A., Kere J. (2012) Identification of 15 new psoriasis susceptibility loci highlights the role of innate immunity. *Nature Genetics,* 44(12):1341-1348.

UCSC "Genome Browser Home". Retrieved from: https://genome.ucsc.edu/ accessed on the 15th of November 2020.

UWCCC Flow Cytometry Laboratory, Titrating Antibodies for Flow Cytometry. Retrieved from: https://cancer.wisc.edu/research/wp-content/uploads/2017/03/Flow_TechNotes_Antibody-Titrations_20170918.pdf accessed on the 3rd of November 2020.

Van Nuffel E., Staal J., Baudelet G., Haegman M., Driege Y., Hochepied T., Afonina I. S., Beyaert R. (2020) MALT1 targeting suppresses CARD14-induced psoriatic dermatitis in mice. *EMBO Journal,* 21(7):e49237.

Vitte J., Michel B. F., Bongrand P., Gastaut J. L. (2004) Oxidative stress level in circulating neutrophils is linked to neurodegenerative diseases. *Journal of Clinical Immunology,* 24(6):683-692.

Volkov M., van Schie K. A., van der Woude D. (2019) Autoantibodies and B cells: The ABC of rheumatoid arthritis. *Immunological Reviews,* 294:148-163.

Wang M., Zhang S., Zheng G., Huang J., Songyang Z., Zhao X., Lin X. (2018) Gain-of-Function Mutation of Card14 Leads to Spontaneous Psoriasis-like Skin Inflammation through Enhanced Keratinocyte Response to IL-17A. *Immunity,* 49(1):66-79.

Wang T., Wei J. J., Sabatini D. M., Lander E. S. (2014) Genetic Screens in Human Cells Using the CRISPR/Cas9 System. *Science,* 343(6166):80-84.

Watson J. D. and Crick F. H. C. (1953) A structure for Deoxyribose Nucleic Acid. *Nature,* 171:737-738.

World Health Organization "Noncommunicable diseases" (1st of June 2018) retrieved from: https://www.who.int/news-room/fact-sheets/detail/noncommunicable-diseases accessed on the 1st of March 2021.

Xu C., Lu Z., Luo Y., Liu Y., Cao Z., Shen S., Li H., Liu J., Chen K., Chen Z., Yang X., Gu Z. & Wang J. (2018) Targeting of NLRP3 inflammasome with gene editing for the amelioration of inflammatory diseases. *Nature Communications,* 9: 4092.

Xu X., Tao Y., Gao X., Zhang L., Li X., Zou W., Ruan K., Wang F., Xu GL., Hu R. (2016) A CRISPR-based approach for targeted DNA demethylation. *Cell discovery,* 2:16009.

Yan J., Cirincione A., Adamson B. (2020) Prime Editing: Precision genome editing by reverse transcriptase. *Molecular Cell,* 77:210-212.

Yao L., Wang H., Song Y., Dai Z., Yu H., Yin M., Wang D., Yang X., Wang J., Wang T., Cao N., Zhu J., Shen X., Song G., Zhao Y. (2017) Large-scale prediction of ADAR-mediated effective human A-to-I RNA editing. *Briefings in Bioinformatics,* 20(1):102-109.

ZhangLab, LentiCRISPRv2 and lentiGuide-Puro: lentiviral CRISPR/Cas9 and single guide RNA, retrieved from: https://media.addgene.org/data/plasmids/52/52961/52961-attachment_B3xTwla0bkYD.pdf accessed on the 23rd October 2020

Zhang S., Wang M., Wang C., Guifen W., Sun K., Xiong S., Cheng L., Yang D., Lin X. and Zhao X. (2021) Intrinsic abnormalities of keratinocytes initiate skin inflammation through the IL-23/T17 axis in a MALT-1-dependent manner. The Journal of Immunology, 206(2) (online

ahead of print) retrieved from: https://doi.org/10.4049/jimmunol.2001031 accessed on the 12th of January 2021.

Zindel J. and Kubes P. (2020) DAMPs, PAMPs, and LAMPs in immunity and sterile inflammation. *Annual Review of Pathology: Mechanisms of Disease,* 15:493-518.

"ADAR", *NCBI,* U.S. National Library of Medicine. Retrieved from: https://www.ncbi.nlm.nih.gov/gene/103 accessed on the 24th of February 2021.

"APOBEC 1", *NCBI,* U.S. National Library of Medicine. Retrieved from: https://www.ncbi.nlm.nih.gov/gene/339 accessed on the 25th of February 2021.

APPENDIX 1. CHOCHOP

Target sequence	Genomic location	Strand	GC content (%)	Self-complementarity	MM0	MM1	MM2	MM3	Efficiency
GAATCCGACGTTAATCTGAAAGG	chr7:80670996	sense	40	0	0	0	0	2	59.56
ACGGCTGCAGGTCAACCTATTGG	chr7:80672799	sense	55	2	0	0	0	4	48.74
GTGCAGAAACAATAGTTGTCTGG	chr7:80671077	antisense	40	1	0	0	0	4	40.14
GATTCCTTTCAGATTAACGTCGG	chr7:80671000	antisense	35	0	0	0	0	6	64.37
GGAAGAACAAATCTATACACAGG	chr7:80671023	antisense	35	0	0	0	0	6	56.85
ACAATTTGCAAAACGGCTGCAGG	chr7:80672787	sense	45	1	0	0	0	6	46.22
TTTCTGATGGCTTGACCAATAGG	chr7:80672814	antisense	40	1	0	0	0	7	42.08
TCGACACATATAAAGGTAAAAGG	chr7:80664476	sense	30	0	0	0	0	7	38.03
ACCTCATTAAGCCAAAGAATAGG	chr7:80673389	antisense	35	0	0	0	0	8	46.89
ACCTTTATATGTGTCGATTATGG	chr7:80664470	sense	30	0	0	0	0	8	30.29
GCCTATTCTTTGGCTTAATGAGG	chr7:80673388	antisense	40	1	0	0	0	9	44.27
GCCATAATCGACACATATAAAGG	chr7:80664469	sense	35	0	0	0	1	8	40.1
GAAATGATCTTACTCAGTGTTGG	chr7:80674061	antisense	35	0	0	0	0	10	57.16
TGCAGAAACAATAGTTGTCTGGG	chr7:80671076	antisense	35	0	0	0	0	10	54.94
TCATTTCTATCAGGCCAAGGAGG	chr7:80674045	antisense	45	1	0	0	0	11	66.56
TTTGGGTTTAATCCATCAATAGG	chr7:80671981	antisense	30	0	0	0	0	11	35.36
GACTCTTAAAACTTGTCTTCAGG	chr7:80671900	sense	35	1	0	0	0	11	24.75
AGATCATTTCTATCAGGCCAAGG	chr7:80674048	antisense	40	1	0	0	0	12	60.06
GTTTTCAACTGGAGAGGCAAAGG	chr7:80671054	antisense	45	1	0	0	2	10	57.1
TCAATAGGTTCTGAAACATCAGG	chr7:80671966	antisense	35	1	0	0	0	13	52.8
TGATTACAGACTGGGACCATTGG	chr7:80673974	sense	45	2	0	0	1	13	59.96
AGAAGAACATAGGACATACTTGG	chr7:80672007	sense	35	1	0	0	1	13	52.27

APPENDIX 2. VBC

Gene name	sgRNA + NGG	Exon number	VBC-score	sgRNA activity 0=bad 1=good	Frameshift ratio inDelphi	Bioscore 0=bad 1=good
CD36	TGAGGAAGTGAAATGTACACAGG	exon 11	0.713	0.778	0.791	0.556
CD36	AAAGAGGTCCTTATACGTACAGG	exon 4	0.762	0.579	0.807	0.83
CD36	AGCCAAGGAAAATGTAACCCAGG	exon 5	0.673	0.716	0.681	0.56
CD36	TGATAGTGAAGGTTCGAAGATGG	exon 5	0.664	0.652	0.662	0.602
CD36	GATTCCTTTCAGATTAACGTCGG	exon 10	0.633	0.641	0.861	0.474
CD36	GGTTCGAAGATGGCACCATTGGG	exon 5	0.659	0.611	0.71	0.611
CD36	AGAAGAACATAGGACATACTTGG	exon 11	0.622	0.493	0.856	0.58
CD36	AACATTCAAGTTAAGCAAAGAGG	exon 4	0.673	0.566	0.889	0.618
CD36	GCCATAATCGACACATATAAAGG	exon 7	0.576	0.262	0.804	0.698
CD36	TCTGTTCCAACTGATAGTGAAGG	exon 5	0.65	0.569	0.58	0.672
CD36	GAAATGATCTTACTCAGTGTTGG	exon 14	0.561	0.57	0.815	0.392
CD36	GATGTTTCAGAACCTATTGATGG	exon 11	0.558	0.488	0.701	0.497
CD36	GAATCCGACGTTAATCTGAAAGG	exon 10	0.548	0.428	0.809	0.49
CD36	CAACTTCACAGTTCTCAATCTGG	exon 5	0.616	0.477	0.639	0.657
CD36	TGATTACAGACTGGGACCATTGG	exon 14	0.553	0.543	0.648	0.457
CD36	ACGGCTGCAGGTCAACCTATTGG	exon 12	0.571	0.602	0.383	0.54
CD36	ACCTCATTAAGCCAAAGAATAGG	exon 13	0.587	0.498	0.658	0.567
CD36	TCGACACATATAAAGGTAAAAGG	exon 7	0.546	0.238	0.87	0.629
CD36	AGGTTCGAAGATGGCACCATTGG	exon 5	0.518	0.36	0.602	0.556
CD36	TGAGTAAGATCATTTCTATCAGG	exon 14	0.509	0.458	0.639	0.438
CD36	ACAATTTGCAAAACGGCTGCAGG	exon 12	0.563	0.469	0.642	0.545
CD36	ATGTTCAGAAGTCAAGTAACTGG	exon 14	0.532	0.519	0.74	0.399
CD36	TGCAGAAACAATAGTTGTCTGGG	exon 10	0.504	0.351	0.694	0.5
CD36	TAGATTTGTTCTTCCATCCAAGG	exon 10	0.503	0.344	0.884	0.437
CD36	ACCTTTATATGTGTCGATTATGG	exon 7	0.524	0.136	0.739	0.716
CD36	ACTGGAAAAATAAACCTCCTTGG	exon 14	0.522	0.601	0.303	0.46
CD36	TCACTTTACAATTTGCAAAACGG	exon 12	0.522	0.346	0.836	0.495
CD36	CACAGTTCTCAATCTGGCTGTGG	exon 5	0.52	0.252	0.648	0.638
CD36	TCATTTCTATCAGGCCAAGGAGG	exon 14	0.518	0.496	0.428	0.498

APPENDIX 3. GPP

sgRNA Sequence	PAM Sequence	Exon Number	Target Cut Leng	Target Total Len	Target Cut %	Off-Target Rank	On-Target Rank	On-Target Rules	On-Target Efficacy Score
GATTCCTTTCAGATTAACGT	CGG	10	847	1419	59.7	8	7	Azimuth_2.0	0.6437
AGGATAAAACAGACCAACTG	TGG	6	592	1419	41.7	10	10	Azimuth_2.0	0.6426
GACCAACTGTGGTAGTAACA	GGG	6	581	1419	40.9	12	14	Azimuth_2.0	0.625
ACTGTGGTAGTAACAGGGTA	CGG	6	576	1419	40.6	14	13	Azimuth_2.0	0.6394
GAATCCGACGTTAATCTGAA	AGG	10	854	1419	60.2	9	19	Azimuth_2.0	0.5956
TCTGTTCCAACTGATAGTGA	AGG	5	375	1419	26.4	24	9	Azimuth_2.0	0.6429
AAAGAGGTCCTTATACGTAC	AGG	4	276	1419	19.5	6	36	Azimuth_2.0	0.5607
TTCACTATCAGTTGGAACAG	AGG	5	388	1419	27.3	47	2	Azimuth_2.0	0.6857
TGGCTTTTCTCAACAAAAGG	TGG	9	768	1419	54.1	17	33	Azimuth_2.0	0.5616
GTCGCAGTGACTTTCCCAAT	AGG	8	715	1419	50.4	4	46	Azimuth_2.0	0.5362
TACCCTGTTACTACCACAGT	TGG	6	590	1419	41.6	32	20	Azimuth_2.0	0.5902
GAAGAACAAATCTATACACA	GGG	10	869	1419	61.2	52	1	Azimuth_2.0	0.7189
TGATAGTGAAGGTTCGAAGA	TGG	5	364	1419	25.7	18	37	Azimuth_2.0	0.5596
TGCAGAAACAATAGTTGTCT	GGG	10	923	1419	65	15	42	Azimuth_2.0	0.5494
TGCGACATGATTAATGGTAC	AGG	8	743	1419	52.4	7	51	Azimuth_2.0	0.5225
TTCGAACCTTCACTATCAGT	TGG	5	380	1419	26.8	3	56	Azimuth_2.0	0.5184
TGATTACAGACTGGGACCAT	TGG	14	1262	1419	88.9	45	17	Azimuth_2.0	0.5996
TAGCAAGTTGTCCTCGAAGA	AGG	4	134	1419	9.4	43	23	Azimuth_2.0	0.5837
TGGGACCATTGGTGATGAGA	AGG	14	1273	1419	89.7	33	34	Azimuth_2.0	0.5615
AACATTCAAGTTAAGCAAAG	AGG	4	260	1419	18.3	64	3	Azimuth_2.0	0.6667
AGCCAAGGAAAATGTAACCC	AGG	5	310	1419	21.8	56	12	Azimuth_2.0	0.64
AGTCACTGCGACATGATTAA	TGG	8	737	1419	51.9	11	62	Azimuth_2.0	0.5038
AGATCATTTCTATCAGGCCA	AGG	14	1325	1419	93.4	60	16	Azimuth_2.0	0.6006
TCATTTCTATCAGGCCAAGG	AGG	14	1322	1419	93.2	75	4	Azimuth_2.0	0.6656
AGAAGAACATAGGACATACT	TGG	11	1108	1419	78.1	31	50	Azimuth_2.0	0.5227
GGAAGAACAAATCTATACAC	AGG	10	870	1419	61.3	50	31	Azimuth_2.0	0.5685
GGAGGTATTCTAATGCCAGT	TGG	3	83	1419	5.8	44	38	Azimuth_2.0	0.5592
ACGGCTGCAGGTCAACCTAT	TGG	12	1171	1419	82.5	19	64	Azimuth_2.0	0.4874

APPENDIX 4. Comparison of all scores

sgRNA + NGG (PAM) [VBC]	Target sequence [CHOPCHOP]	sgRNA Sequence [DOENCH]
TGAGGAAGTGAAATGTACACAGG	GAATCCGACGTTAATCTGAAAGG	GATTCCTTTCAGATTAACGTCGG
AAAGAGGTCCTTATACGTACAGG	ACGGCTGCAGGTCAACCTATTGG	AGGATAAAACAGACCAACTGTGG
AGCCAAGGAAAATGTAACCCAGG	GTGCAGAAACAATAGTTGTCTGG	GACCAACTGTGGTAGTAACAGGG
TGATAGTGAAGGTTCGAAGATGG	GATTCCTTTCAGATTAACGTCGG	ACTGTGGTAGTAACAGGGTACGG
GATTCCTTTCAGATTAACGTCGG	GGAAGAACAAATCTATACACAGG	GAATCCGACGTTAATCTGAAAGG
GGTTCGAAGATGGCACCATTGGG	ACAATTTGCAAAACGGCTGCAGG	TCTGTTCCAACTGATAGTGAAGG
AGAAGAACATAGGACATACTTGG	TTTCTGATGGCTTGACCAATAGG	AAAGAGGTCCTTATACGTACAGG
AACATTCAAGTTAAGCAAAGAGG	TCGACACATATAAAGGTAAAAGG	TTCACTATCAGTTGGAACAGAGG
GCCATAATCGACACATATAAAGG	ACCTCATTAAGCCAAAGAATAGG	TGGCTTTTCTCAACAAAAGGTGG
TCTGTTCCAACTGATAGTGAAGG	ACCTTTATATGTGTCGATTATGG	GTCGCAGTGACTTTCCCAATAGG
GAAATGATCTTACTCAGTGTTGG	GCCTATTCTTTGGCTTAATGAGG	TACCCTGTTACTACCACAGTTGG
GATGTTTCAGAACCTATTGATGG	GCCATAATCGACACATATAAAGG	GAAGAACAAATCTATACACAGGG
GAATCCGACGTTAATCTGAAAGG	GAAATGATCTTACTCAGTGTTGG	TGATAGTGAAGGTTCGAAGATGG
CAACTTCACAGTTCTCAATCTGG	TGCAGAAACAATAGTTGTCTGGG	TGCAGAAACAATAGTTGTCTGGG
TGATTACAGACTGGGACCATTGG	TCATTTCTATCAGGCCAAGGAGG	TGCGACATGATTAATGGTACAGG
ACGGCTGCAGGTCAACCTATTGG	TTTGGGTTTAATCCATCAATAGG	TTCGAACCTTCACTATCAGTTGG
ACCTCATTAAGCCAAAGAATAGG	GACTCTTAAAACTTGTCTTCAGG	TGATTACAGACTGGGACCATTGG
TCGACACATATAAAGGTAAAAGG	AGATCATTTCTATCAGGCCAAGG	TAGCAAGTTGTCCTCGAAGAAGG
AGGTTCGAAGATGGCACCATTGG	GTTTTCAACTGGAGAGGCAAAGG	TGGGACCATTGGTGATGAGAAGG
TGAGTAAGATCATTTCTATCAGG	TCAATAGGTTCTGAAACATCAGG	AACATTCAAGTTAAGCAAAGAGG
ACAATTTGCAAAACGGCTGCAGG	TGATTACAGACTGGGACCATTGG	AGCCAAGGAAAATGTAACCCAGG
ATGTTCAGAAGTCAAGTAACTGG	AGAAGAACATAGGACATACTTGG	AGTCACTGCGACATGATTAATGG
TGCAGAAACAATAGTTGTCTGGG	TGAGTAAGATCATTTCTATCAGG	AGATCATTTCTATCAGGCCAAGG
TAGATTTGTTCTTCCATCCAAGG	GTCTGGGTTTTCAACTGGAGAGG	TCATTTCTATCAGGCCAAGGAGG
ACCTTTATATGTGTCGATTATGG	ACTATATTGTGCCTATTCTTTGG	AGAAGAACATAGGACATACTTGG
ACTGGAAAAATAAACCTCCTTGG	TAGATTTGTTCTTCCATCCAAGG	GGAAGAACAAATCTATACACAGG
TCACTTTACAATTTGCAAAACGG	GTTGTACTGAAATATATGTATGG	GGAGGTATTCTAATGCCAGTTGG
CACAGTTCTCAATCTGGCTGTGG	TTATTAACTTGATTACAGACTGG	ACGGCTGCAGGTCAACCTATTGG
TCATTTCTATCAGGCCAAGGAGG	TCAATAAGGTGTTTTCTTACAGG	ACTTTGAGAGAACTGTTATGGGG