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- Our future with CRISPR: a brave new world?
- CRISPR/Cas9 gene editing as a cancer therapy

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FROM THE EDITORIAL BOARD

Dear reader,

After a turbulent 2020, 2021 has officially started. The past year has been challenging for all of us in many different aspects, and, unfortunately, not much seems to be different at the start of 2021. Quite fitting - I am writing this introduction on Blue Monday. However, besides these negative talks, I believe it is (almost) always possible to be thankful for something. My outlook might be a bit biased, considering that I am an inveterate optimist. Nevertheless, I am happy about the free bouquet I received at the supermarket today because of Blue Monday, the fact that we are nearing spring again, and the stunning nature around Nijmegen. Moreover, I am grateful for this edition of RAMS. We owe a debt of gratitude to our editors, reviewers, and students who submitted their article, who found the time to dive into a wide range of topics to write about or to review all of this edition's articles.

In this edition we will address two of the Nobel Prizes awarded in 2020. What are the possible (future) applications of CRISPR/Cas9 within healthcare and other fields? Why did Michael Houghton, Harvey Alter, and Charles Rice receive the Nobel Prize in Physiology or Medicine for their discovery of hepatitis C? Furthermore, we will dive into thrombocytopenia in our Zebras of Medicine: when is it a sign of immune thrombocytopenic purpura and when of liver cirrhosis? Next, we will discuss the possible underdiagnosis of females with ADHD in our Myth or Science. Other topics include the translation of vaccine research from mice to humans, the plasticity of the human brain, longevity, and gender and sex bias in biomedical research. I would say that we have a topic for every taste. We hope that you enjoy reading this edition!

Yours faithfully,

Yfke Prins, Editorial Editor-in-Chief

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"WHY WERE HARVEY ALTER, MICHAEL HOUGHTON, AND CHARLES RICE AWARDED THE NOBEL PRIZE FOR THEIR DISCOVERY OF THE HEPATITIS C VIRUS?"

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Abstract

The 2020 Nobel Prize in Physiology or Medicine was jointly awarded to three scientists—Harvey J. Alter, Michael Houghton, and Charles M. Rice—for their discovery of the hepatitis C virus. Their work made a paramount impact on blood-borne hepatitis, a global health threat that can cause cirrhosis and different liver cancers in infected patients. The prior identification and characterisation of hepatitis A and B viruses were vital; however, a large number of hepatitis cases could not be explained. The discovery of the hepatitis C virus by these scientists aided in explaining the majority of the remaining chronic hepatitis cases and in the rapid development of diagnostic blood tests and antiviral drugs that have saved millions of lives and greatly reduced the burden on healthcare systems. The world is currently presented with an opportunity to eradicate hepatitis C virus infections altogether. However, exceptional international collaboration and financing are needed to implement widespread diagnostic testing and distribution of antiviral drugs to rid the world of the hepatitis C virus.

KEYWORDS: hepatitis C virus; Nobel Prize; antiviral drugs; infectious diseases

Hepatitis, commonly known as liver inflammation, is typically caused by viral infections, although other factors such as alcoholism, environmental toxins, and certain autoimmune conditions can contribute to its aetiology as well [1, 2]. The discovery of hepatitis viruses is one of the most important scientific breakthroughs of the past five decades. In the second half of the 20th century, two different hepatitis viruses, namely the hepatitis A and B virus, were identified [3, 4]. The hepatitis A virus is known to infect people through the ingestion of the virus in contaminated food and water, seldom causing severe long-term effects [5]. It is a highly contagious virus whose spread is now controlled in most countries by the introduction of a vaccine in 1995 [5]. The hepatitis B virus is transmitted through infected blood and other bodily fluids and is considered a more serious disease as it can stay dormant for long periods, after which it can induce liver cirrhosis and cancer [5]. Blood-borne hepatitis is a major concern for global health, annually causing over a million deaths worldwide, comparable to other serious conditions such as tuberculosis and HIV-AIDS [6]. Although the discovery of hepatitis A and B explained the cause of many hepatitis cases, a large number remained unexplained [7]. This article describes the work of three scientists that led to the discovery of the hepatitis C virus, which explained these infections, placing the world in a position to eventually eliminate viral hepatitis [8].

A mysterious viral agent

The detection and characterisation of infectious agents are pivotal for identifying disease outbreaks, for developing effective intervention strategies, and for epidemiological studies. Jaundice, a striking symptom common to hepatitis infections, was known since antiquity [3]. Epidemics of jaundice were a menace during the two world wars, and the cause was attributed to poor sanitary living conditions of soldiers [3]. Although the cause of these cases was traced back to the hepatitis A virus, it was only after the identification of the hepatitis B virus and the advent of novel molecular biology techniques that scientists identified the hepatitis A virus in stool samples [3]. Hepatitis A is now known to be an RNA virus belonging to the family *Picornaviridae*, transmissible through contaminated food and water [3]. The spread of the hepatitis A virus is greatly limited by the introduction of a vaccine in 1995 [5].

In 1967, Dr. Baruch Blumberg and his colleagues discovered the hepatitis B virus by finding significant amounts of the hepatitis B surface antigen, formerly termed the "Australia antigen", in the serum of an Aboriginal Australian [9]. The identification of this antigen resolved problems faced by scientists and healthcare providers for decades—the absence of a specific biomarker to distinguish between different types of viral hepatitides [10]. Two years later, the hepatitis B vaccine was invented, and Dr. Blumberg was awarded the Nobel Prize in Physiology or Medicine for his discovery [11]. Along with aiding physicians in the diagnosis of asymptomatic carriers of the virus, the safety of blood transfusions was greatly improved [12].

Around the same time, Harvey J. Alter was studying the incidence of hepatitis in patients who had received blood transfusions at the National Institute of Health. After performing diagnoses for the newly discovered hepatitis A and B viruses, Alter and his colleagues showed that a large proportion of cases remained unexplained [7]. In 1978, the same group demonstrated that the blood from these hepatitis A and B negative patients was able to infect chimpanzees and cause an increase in the levels of biomarkers associated with typical liver inflammation [13]. This unknown disease was appropriately named "non-A, non-B" hepatitis, a novel, severe form of hepatitis whose aetiology was yet to be determined. Assays involving chloroform inactivation, electron microscopy, and filtration studies all indicated the causative agent to be an enveloped virus of about 45-60 nm in diameter [14]. However, conventional cultivation experiments and serology approaches failed, partly due to the low amount of viral antigen in infected sera [14]. Scientists were frustrated for 15 years until the causative agent was finally identified and characterised as the hepatitis C virus due to the collective efforts of three scientists—Harvey Alter, Michael Houghton, and Charles Rice [8].

Discovery of the Hepatitis C Virus

After the establishment of the novel "non-A, non-B" viral hepatitis in post-transfusion patients by Alter and colleagues, the identification and characterisation of the unknown virus was of grave importance. As all conventional isolation strategies failed, the virus evaded detection for more than a decade until 1989, when Michael Houghton—a scientist at

"Why were Harvey Alter, Michael Houghton, and Charles Rice awarded the Nobel Prize for their discovery of the hepatitis C Virus?" - Ramu

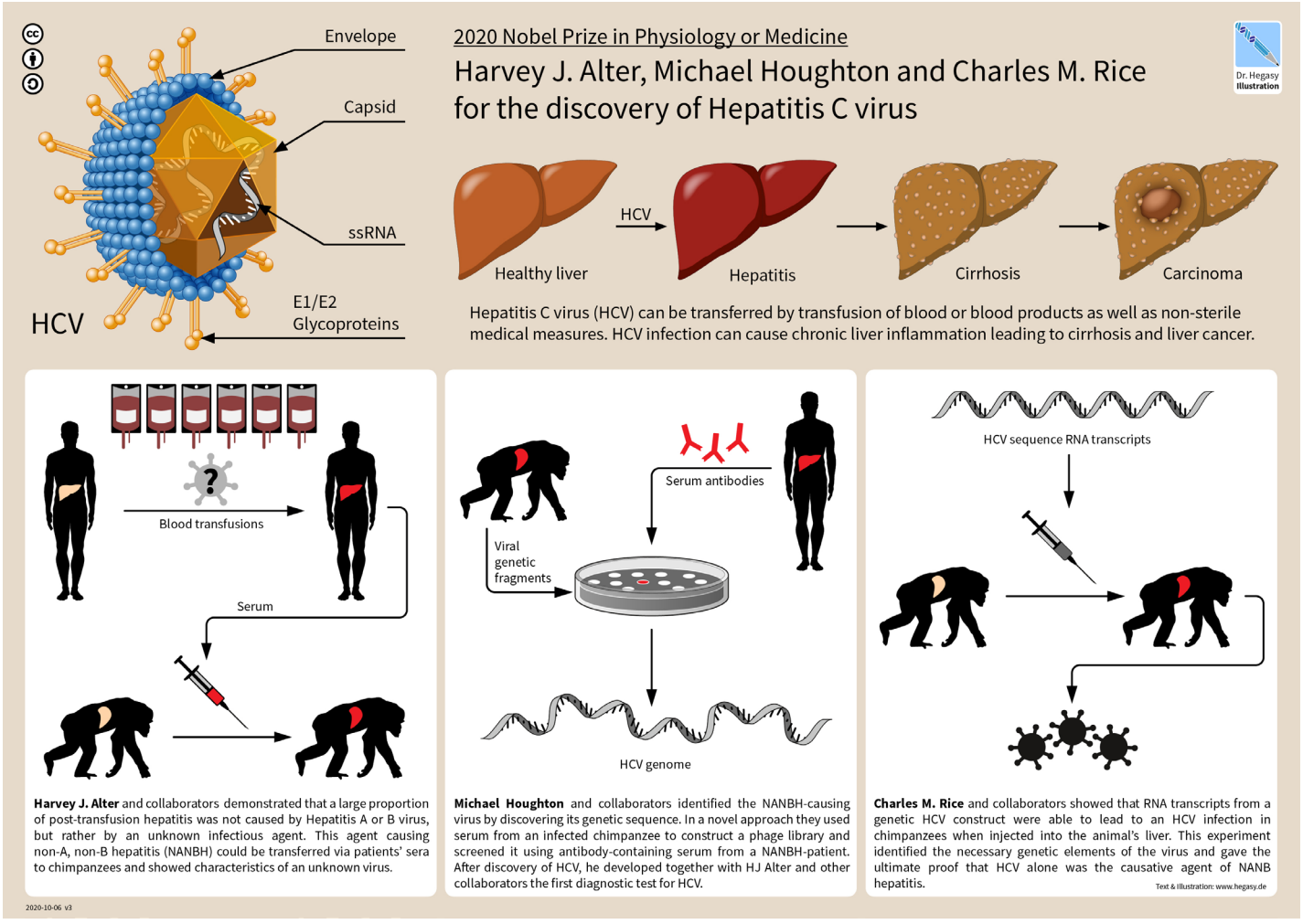

Figure 1: A summary of three key discoveries by Harvey J. Alter, Michael Houghton, and Charles M. Rice that led to the discovery of the hepatitis C virus. *Illustration: www.hegasy.de*

the pharmaceutical company Chiron—isolated the genetic sequence of the virus [15]. Houghton and his colleagues collected and curated a library of cDNA fragments, isolated from the blood of a chronically infected chimpanzee [15]. The rationale behind their experiments was to exclude fragments derived from the genome of the chimpanzee to isolate and identify the fragments that originated in the uncharacterised "non-A, non-B" hepatitis virus. The group achieved this by using antibodies isolated from the sera of chronically infected patients to identify the rare clones of DNA that encoded viral proteins [15]. After a series of rigorous experiments, one positive clone was identified to contain the partial sequence of the agent responsible for the mysterious infections [15]. Further research indicated that it was derived from an enveloped, positive-sense, single-stranded RNA molecule of approximately 10,000 nucleotides in length, belonging to the family of *Flaviviruses* [15]. The detection of antibodies in chronic "non-A, non-B" hepatitis patients specific to this newly discovered virus further validated the findings [16]. The novel virus was given the name "hepatitis C virus", and the molecular techniques used for its identification were recognised as potential approaches for the identification of other unknown infectious agents as well [17]. After this revolutionary discovery, the first diagnostic test for the hepatitis C virus was developed [18].

Although the discovery of the hepatitis C virus was conclusive, a crucial question remained: could the virus alone replicate and cause "non-A, non-B" hepatitis? This question was answered by scientist Charles M. Rice and colleagues at Washington University. Previously, the researchers observed an uncharacterised genomic region at the end of the viral genome that they speculated to be important for its replication [19]. Rice and his colleagues also detected specific genomic mutations in the virus isolated from patient samples that he hypothesised to prevent efficient viral replication [19]. Therefore, Rice and his colleagues genetically engineered a hepatitis C virus RNA transcript, excluding the debilitating genetic variants [19]. On injection of the construct directly into the livers of the chimpanzees, viral titres were detected in the blood, and pathological features similar to those observed in chronically infected human patients were observed [19]. These crucial experiments identified the genetic elements essential for viral replication and provided the missing piece of the puzzle that the hepatitis C virus alone was able to establish the "non-A, non-B" hepatitis observed in patients receiving blood transfusion [19].

A remarkable Nobel Prize-winning discovery

The discovery of hepatitis C viruses earned Harvey Alter, Michael Houghton, and Charles Rice the 2020 Nobel Prize in Physiology or Medicine [8]. Their findings are hailed as a scientific milestone in the ongoing battle against viral diseases (Figure 1) [8]. Owing to their work, highly specific and sensitive diagnostic blood tests have been developed, which have been instrumental in eliminating post-transfusion hepatitis in numerous countries worldwide [20].

An average of three to four million people gets infected with the hepatitis C virus annually, rendering them susceptible to cirrhosis, liver failure,

and hepatocellular carcinoma [21]. The novel discoveries provided a much-needed foundation with which efficient antiviral drugs have been developed [14]. These antiviral drugs are well-tolerated and are able to cure the disease; thus, these drugs provide the opportunity to save lives, reduce healthcare costs, and rid the world of the hepatitis C virus [14]. However, to reach this goal, collaborative international efforts are needed to advance widespread blood testing, to produce antiviral drugs at a large scale, and to distribute them across the globe.

Although the World Health Organization aims for the eradication of the hepatitis C virus by 2030, a progress report in 2020 indicated that merely one in five people with hepatitis C is diagnosed and even less receive treatment and are cured [22]. Lack of adequate financing remains the sole barrier to achieve widespread testing and treatment [22]. Therefore, the commitment of philanthropic funders and investments from public-private partnerships with industry are urgently needed to finish the work started by the Nobel Prize winners [22]. Increased financing can also aid in catalysing certain developing countries to re-assess and improve their health care programs to eliminate hepatitis C virus circulation [22]. Current large-scale investments and extensive international collaboration for COVID-19 suggests that it should be possible to mobilise resources to rid the world of the hepatitis C virus [23].

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OUR FUTURE WITH CRISPR: A BRAVE NEW WORLD?

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Imagine a world without malaria, without transplant shortages, and even without genetic diseases. A world where climate change has been halted or reversed. A world where you can design your baby to be the most intelligent, the most creative, and the most courageous. Would you want to live in that world? It may sound like science fiction, but this question is becoming more and more relevant. It is the field of genome engineering that holds all these great promises. Through genome engineering, the DNA of any living organism can be changed to give it the desired characteristics. Mosquitoes that no longer transmit malaria, pigs that grow human-compatible organs, and elephants that resist the cold, just like the woolly mammoth, are all on their way. While genome engineering finds its origins in the previous century, the discovery of CRISPR/Cas9 has enabled rapid progress. But how does this process of genome editing actually work? And, what can and what should we do with this powerful tool?

In 2020, the Nobel Prize in Chemistry was awarded for the 112th time. Yet, it was the first time for the prize to be awarded to two women. Emmanuelle Charpentier and Jennifer Doudna received the prize for discovering "the sharpest tool in gene technology: the CRISPR/Cas9 genetic scissors" [1]. With CRISPR/Cas9, the DNA of animals, plants, and microorganisms can be changed with high precision. In less than ten years from this discovery, CRISPR/Cas9 has become famous for its (promised) applications, ranging from genetically modified foods to the treatment of genetic disease and even to designer babies (Figure 1). To better understand the potential as well as the dangers of CRISPR/Cas9, we will discuss its discovery, mechanism, and (future) applications.

It all started with CRISPR...

The story of CRISPR/Cas9 is a detective story on mysterious microbial defence systems that were developed into the most powerful tool to engineer genetic information. It started in 1987 when studies in *E. coli* bacteria reported remarkable repeats in the DNA, which were short and palindromic [2]. A palindrome is a word or sequence that reads the same backwards as forwards, such as *rotator*, or *ATTA* for a DNA sequence. In certain clusters of the bacterial DNA, the same sequence was repeated over and over, but with variable pieces of DNA, called 'spacers', in between. The same repeats were found in many other bacteria; however, their function remained unknown [3]. Only fifteen years later, the repeats were given the name "CRISPR" for Clustered Regularly Interspaced Short Palindromic Repeats [4]. Yet, it required a change of focus towards the spacers to finally unravel its function. As it turned out, the spacers matched the DNA of bacteria-specific viruses, called bacteriophages. [5]. Upon infection with bacteriophages, the bacteria incorporate a part of the bacteriophage DNA into their own DNA as a variable spacer between the repeats [6]. This process gives the bacteria a memory of their infections as a form of adaptive immunity. Next time, when a bacteriophage infects the bacteria, those that previously acquired the matching spacer DNA will survive the infection.

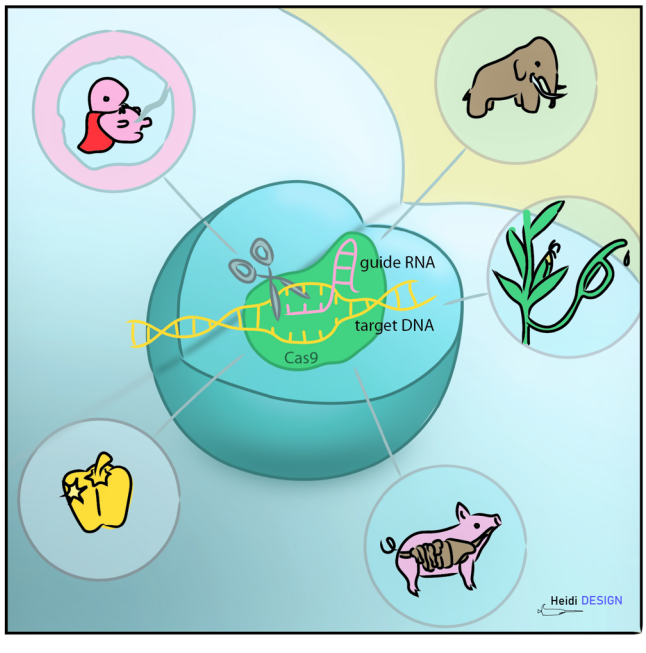

Figure 1: The CRISPR/Cas9 genetic scissors can be used to cut DNA. Through genome editing, superfoods, biofuels, mammoths, xenotransplants, and CRISPR babies can be engineered.

... then came Cas9

To unravel the mechanisms by which these spacers, also called memory sequences, of the bacteria can provide immunity, researchers turned their attention to the neighbouring DNA. Here, they discovered that the bacteria also have genes for the CRISPR-associated (Cas) enzymes. It turned out that the memory sequences are transcribed from DNA into RNA and that this memory RNA guides the Cas enzymes [7]. This bacterial CRISPR/Cas complex, subsequently, cleaves the matching bacteriophage DNA [7]. Since 2010, we, therefore, know that the Cas9 enzyme is an "RNA-guided DNA nuclease", guided by the memory RNA to destroy the bacteriophage DNA [7].

Genetic scissors

But what brings us from bacteria cleaving their invaders to genome engineering? That is where Emmanuelle Charpentier and Jennifer Doudna come into play. These scientists were the first to understand the Cas9 mechanisms well enough to take them out of the bacteria and put them in a test tube. Charpentier and Doudna showed in these test tubes that the Cas9 activity is programmable; if you give the Cas9 enzyme the right guide RNA (gRNA), which is an artificially designed memory RNA, it can cleave any DNA of interest [8]. Therefore, Charpentier and Doudna could make Cas9 cleave not only bacteriophage DNA but also that of animals [9, 10].

The experiments of Charpentier and Doudna showed that the Cas9 enzyme provides the long-dreamed-of programmable and precise "genetic scissors" [8]. With the right gRNA, any specific piece of DNA, whether human, animal, plant, or microbial, can be cut. The broken DNA will recruit the repair machinery of the cell [11]. Usually, the DNA strand break will be repaired with small changes (non-homologous end-joining) [11]. However, these repair mechanisms can introduce crucial sequence errors, leading to gene inactivation [11]. In some cases, the DNA will be repaired by using the second copy of DNA in the cell as a template (homology-directed repair) [11]. If we provide the cell with a false copy of DNA that looks very similar but contains changes engineered by us, this template will introduce the preferred changes in the genome [11]. Through these options, the Cas9-induced DNA strand break enables us to rewrite the DNA [11]. Wherever the genetic scissors cut, we can add, delete, or replace DNA [11].

Rewriting the code of life

The CRISPR/Cas9 genetic scissors have started a revolution in genome engineering. Now that we can rewrite the DNA code of any form of life, a whole new world of opportunities has opened up. From food production to climate change to public health, many emerging problems could be solved, or at least eased, through genome engineering.

Food production

The genetic engineering of any organism with agricultural or industrial significance could enhance the quality and quantity of our food. Many important candidates can be named, ranging from microbes to plants to animals. For example, microbes in the soil could be engineered to improve the soil quality, thereby increasing the harvest [12]. Agricultural crops could be enhanced such that they are resistant to environmental disturbances or pathogenic infections, or such that the produced food is healthier [13]. For instance, improved rice varieties in which the genes for metal absorption have been edited, are now available [14]. These rice varieties contain lower levels of toxic cadmium and arsenic. Crops that can resist pests offer opportunities to lower the use of pesticides [15].

Climate change

Genetic engineering could also be used in our battle to mitigate or even stop global warming. Bacteria producing the right chemical compounds represent a sustainable source of biofuels [16]. Through genetic engineering, the relevant metabolic pathways may be enhanced towards increased production of the biofuel compounds. A less conventional example is given by the "mammoth project" from Harvard [17]. This group wants to bring the extinct woolly mammoth back to life, or at least they want to bring specific genes, such as those conferring cold-resistance, from the woolly mammoth back into the elephant. Apparently, the woolly mammoths played an important role in limiting methane release (an important greenhouse gas) into the air. If the cold-resistant elephants could do the same, they would contribute to the stabilisation of climate change.

Health

CRISPR/Cas9 also has many applications in biomedicine and public health. To improve our health, genetic engineering can be used directly on human cells or those of organisms that could threaten or ameliorate our health. For example, gene drives with CRISPR/Cas9 could be used to stop the transmission of malaria and other mosquito-transmitted diseases [18, 19]. In such gene driven approaches, genetic modifications are used that either kill mosquitoes or make them unable to transmit the pathogens. As malaria alone kills more than 400,000 individuals per year, this would have great health benefits [20]. Another example is xenotransplantation, where organs from other species, such as pigs, would be transmitted to human patients [21]. This could solve the big problem of transplant waiting times that are a considerable burden on the quality of life and survival chances of patients. The donating animals would be genetically modified to make the xenotransplant organs suitable for transplantation to humans. According to the latest developments, up to 62 pig genes could be edited to increase the immunocompatibility of pig organs [22].

The use of CRISPR/Cas9 in humans offers ample opportunities in the treatment and prevention of disease. Important steps have already been taken in the field of engineered therapeutic cells. For example, chimeric antigen receptor T-cells are T-cells from the patient that are genetically modified *ex vivo* to attack the cancer cells and then infused back into the patient [23]. These CAR T-cells can efficiently recognise the cancer cells through the genetic insertion of the right T-cell receptor sequences. *In vivo* genome engineering is technically more challenging than these *ex vivo* modifications. Yet, if delivery and editing are efficient enough, *in vivo* approaches could offer a life-long cure for certain genetic diseases otherwise requiring life-long medicine intake for symptom alleviation [24]. High up the priority list are monogenic diseases, such as cystic fibrosis and spinal muscular atrophy, where genetic engineering could correct the causative mutation [25, 26]. Over the last years, the first successes have been reported, for example, for patients with sickle cell disease [27].

Designer babies

Probably the most infamous application of genome engineering is that of "designing babies". While engineering of human germline cells or embryos is strictly forbidden in most countries, the upcoming practices of pre-implementation genetics and selection illustrate the wish of some parents to choose for a child without 'detrimental' or even with more 'beneficial' gene variants [28, 29]. Genome engineering might replace this selection in the future, as only one embryo would then be needed to edit genes associated with the demanded characteristics. Next to the correction of disease mutations, such as cystic fibrosis and sickle cell disease, novel, beneficial variants could be edited. For example, mutations in the gene encoding for the C-C chemokine receptor type 5 (CCR5) receptor prevent the human immunodeficiency virus (HIV) from entering cells, thereby offering resistance to this infection [30]. It is exactly this gene that was edited in the first CRISPR babies [31]. When the first reports on these CRISPR babies, two twin girls from China, came out in November 2018, the global debate on the controversial aspects of CRISPR/Cas9 intensified. Not only was this the first time when the genetic code of a future generation was edited, but it also concerned a gene that is somewhat in the rather grey area between disease prevention and human enhancement. Candidate genes for such enhancement include the *myostatin* gene, which is essential for muscle growth and the *basic helix-loop-helix family member e41* gene, for which certain variants reduce the amount of sleep that you need [32, 33]. But who would decide which and how many genes we are allowed to edit? Will only rich people be able to afford this, thereby increasing the gap between the rich and the poor? And will your genetic profile then be screened as a requirement for certain jobs?

Ethical and moral considerations

The sometimes-horrifying consequences that genetic engineering might have are widely presented to us in science-fiction books and movies. Examples include Jurassic Park ("Genetic power is the most awesome force the planet's ever seen, but you wield it like a kid that's found his dad's gun") and genetic discrimination in the movie *Gattaca* ("a new underclass, no longer determined by social status or the color of your skin. No, we now have discrimination down to a science."). Opponents clearly state that gene editing of germline cells and embryos should not be allowed, not under any circumstances [34, 35]. The unpredictable effects on future generations would make such use dangerous and ethically unacceptable. Furthermore, opponents believe that we should prevent a "brave new world", where a kid would be manufactured to

play a specific role in society. Proponents argue the opposite and state that it is not ethical to longer "roll the dice with our kids' lives" [36]. They state that applying CRISPR/Cas9 in adult patients is not enough, as their children will still inherit the disease genes, and that it would be unethical not to put an end to this preventable suffering and death [34].

Conclusion

The story of CRISPR/Cas9, a microbial defence system that turned into powerful genetic scissors, seems to only just have started. By rewriting the code of life, genetic engineering opens up opportunities in food production, climate change stabilisation, and public health. A long road lies ahead, both promising and troublesome. However, while technical challenges continue to be addressed, many ethical challenges remain unsettled. And, while certain CRISPR/Cas9 applications are undoubtedly beneficial, others are alarming. The central question is no longer what we *could*, but rather what we *should* use these powerful genetic scissors for. Now that the future of humanity, and that of all other organisms, lies in our hands, we must act carefully and responsibly. Scientists, ethicists, politicians, and policymakers all must be involved in the debate on regulation and surveillance. Only with societal support and safety requirements can CRISPR/Cas9 indeed change our world for the better.

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THE POTENTIAL OF CRISPR/CAS9 GENE EDITING AS A CANCER THERAPY BY TARGETING FUSION GENES

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Abstract

The CRISPR/Cas9 genome editing system might hold potential as a cancer therapy. Furthermore, fusion genes are an attractive target for cancer genome editing as they are generally restricted to cancer cells. Currently, two cancer genome editing strategies using CRISPR/Cas9 have been published, of which one involves a partial gene deletion relying on non-homologous end-joining for DNA repair and one that involves a suicide gene addition relying on homology-directed repair. This paper outlines and compares these two strategies, including the current challenges still faced by cancer genome editing. While the suicide gene addition strategy has a wider scope of potential fusion gene targets, the partial gene deletion strategy allows for better standardisation of a potential therapeutic and relies on the more efficient non-homologous end joining, thus making the partial gene deletion strategy a more attractive therapy. However, further research into safety, efficacy, and a comparison with the current standard of care is necessary to fully evaluate the potential of CRISPR/Cas9 genome editing as a cancer therapeutic.

The discovery of the CRISPR/Cas9 genome editing system has allowed for a simple and specific way to edit the genome. The CRISPR/Cas9 system has been used in successful clinical trials to alter the genome of human cells to treat genetic disorders, both *in vivo* and *ex vivo* [1]. Furthermore, in the future, CRISPR/Cas9 genome editing might also be harnessed as a therapy to treat cancers. When treating cancer through genome editing, off-target genome editing of healthy cells should be avoided. One way of making genome editing specific for cancer cells is by choosing a genotype-specific target for cancer cells. Recently, two different cancer genome editing approaches have been published using fusion genes as the genotype-specific target [2, 3]. This paper will review these two approaches, compare their strengths and weaknesses, and review the current challenges both approaches still face.

CRISPR/Cas9 genome editing

The CRISPR/Cas9 system is a genome editing technology that allows for precise cutting in the DNA by the Cas9 enzyme. The Cas9 enzyme is a nuclease that is guided to a particular location in the genome by a guide RNA (gRNA), where it will then produce double-stranded breaks (DSBs) [4]. In gene editing, these DSBs can be used to delete a stretch of nucleotides or as a place to insert new genetic material [5].

After the induction of the DSBs by Cas9 enzymes, the cell will repair the cuts mainly via the two following pathways: non-homologous end-joining (NHEJ) and homology-directed repair (HDR). In NHEJ, a re-ligation of the two strands occurs. However, this process is error-prone and will often lead to small insertions and deletions—also known as indels—at the site of the DSB [6]. In contrast to the error-prone NHEJ, the DSB can also be repaired through HDR in which a template is used. In a physiological setting, this template is the sister chromatid, allowing for error-free repair of the DSB [4]. NHEJ can occur in all phases of the cell cycle, whereas HDR can only occur during the late S- or G2 phase when a sister chromatid is present [7]. In most cases, NHEJ is favoured over HDR, and in non-dividing cells, NHEJ is the only option [8]. However, different strategies exist to increase the efficiency of HDR over NHEJ [8, 9].

The CRISPR/Cas9 gene-editing approaches rely on DNA repair through NHEJ or HDR to either knock out or knock-in a gene. A single DSB that is repaired through the NHEJ pathway will usually lead to indels, which can cause a frameshift in the sequence, often leading to a knockout of the gene, also referred to as gene disruption [5]. Another way to knock out a gene also relies on a larger deletion of the gene through NHEJ. In this case, instead of one DSB, two DSBs are induced to flank the gene, which often leads to the removal of the DNA lying in between and results in the re-ligation of the DNA strands flanking the gene [10]. Additionally, the DNA flanked by the DSBs might be re-ligated at the opposite ends, leading to an inversion of the DNA sequence, which will also often lead to a knockout of the gene [10]. Knocking-in of genes, also known as gene addition, on the other hand, introduces new DNA into the genome and relies on the HDR pathway. During this process, the Cas9 enzyme can be delivered together with a recombination donor—a stretch of the DNA that needs to be built in, for example, a new gene [9]. In the recombination donor, the desired sequence is flanked by homologous regions, inducing the HDR mechanism, leading to a repaired DSB containing the desired new sequence [7, 9]. Additionally, by inducing two DSBs instead of one, it is possible to further increase the efficiency of HDR and the use of the recombination donor [10].

Fusion genes

Both Martinez *et al.* and Chen *et al.* used fusion genes as a target site for cancer genome editing [2, 3]. Fusion genes are especially prevalent in cancer cells as the genomic instability associated with cancer results in increased chromosomal rearrangements, leading to the creation of fusion genes [11]. A fusion gene can occur when, for example, a chromosomal translocation causes two previously independent genes to be located next to each other at the breakpoint [12]. The two joined gene partners of the fusion gene are transcribed together, resulting in a fusion protein which in some cases can have oncogenic properties [12]. Furthermore, the creation of fusion genes that involve tumour suppressor genes might lead to decreased expression of the tumour suppressor, again promoting oncogenesis [13]. Overall, a fusion gene might lead to a loss of function, a loss of regulation, or completely new properties [14]. Fusion genes

with oncogenic properties will confer a survival advantage to the tumour cells, making oncogenic fusion genes more likely to reoccur [14]. Another source that provides evidence that fusion genes are an auspicious target is the recent analysis of Gao *et al.* concerning tumour RNA sequencing data [13]. This study suggests that fusion genes play a driving role in 16.5% of all cancer cases, making the group of patients that potentially benefit from fusion gene-targeted therapy not unsubstantial [13]. As these fusion genes are restricted to cancer cells, they make an attractive target for cancer therapy, and small molecule inhibitors of fusion proteins have been successfully used as targeted therapy [15]. Other approaches to target these fusion genes are also under investigation, including the possibility of CRISPR/Cas9 based genome editing as shown by the approaches taken by Martinez *et al.* and Chen *et al.* [2, 3].

Targeted CRISPR/Cas9 editing of fusion genes

While both Martinez *et al.* and Chen *et al.* approached cancer genome editing by using fusion genes as a target, Martinez *et al.* used a strategy that relied on a gene deletion, while Chen *et al.* used a strategy that relied on gene addition [2, 3].

In the approach taken by Martinez *et al.*, CRISPR/Cas9 genome editing was used to perform a gene knockout of a targeted fusion gene, thereby removing its oncogenic effects (Figure 1a). Two DSBs were induced to remove the activity of the fusion gene, one in each gene partner of the fusion gene, flanking the breakpoint [3]. As a consequence of the DSBs, part of the DNA sequence of the fusion gene was deleted or inverted, thereby knocking out the fusion gene [3]. Importantly, this strategy targets explicitly cancer cells because these cells harbour the fusion gene; healthy cells with no fusion gene are unaffected [3]. The large deletion or inversion of a part of the fusion gene is only likely to occur when the gene partners have rearranged into a fusion gene, as only then the two DSBs are located on the same chromosome [3]. While DSBs can occur in healthy cells, the gRNAs located the DSBs in intronic regions; thus, the small indels generated by NHEJ would not affect the expression of the non-rearranged gene partners [3].

In contrast, Chen *et al.* did not knock out the activity of the fusion gene but instead merely used the fusion gene as a cancer-specific location for the addition of the herpes simplex virus type 1 thymidine kinase *(HSV1-tk)* gene, a suicide gene (Figure 1b). This type of gene produces a protein that will convert a non-toxic compound (the prodrug) into a toxic compound (the active drug), leading to cell death [16]. The HSV1-tk protein can phosphorylate the prodrug ganciclovir, and in cells expressing HSV1-tk, ganciclovir can be converted to ganciclovir triphosphate [17]. DNA polymerase can incorporate ganciclovir triphosphate into new DNA, which causes chain termination and, subsequently, cell death [17]. Cells that do not express HSV1-tk do not experience this effect as ganciclovir cannot be phosphorylated [2].

The insertion of the suicide gene was achieved by designing two gRNAs to allow for a cut in each gene partner flanking the breakpoint, and, additionally, a recombination donor sequence that included the HSV1-tk cDNA was delivered [2]. The recombination donor included homologous sequences of both gene partners to allow for HDR-mediated gene addition, as these homologous sequences promote engagement of HDR [7]. Furthermore, to allow for correct RNA splicing of the HSV1-tk mRNA, the suicide gene was flanked by a splice acceptor and a splice donor [2]. The suicide gene did not have a promoter but did contain sites for independent translation initiation, allowing for the production of the HSV1-tk protein from the fusion gene RNA [2]. While Martinez *et al.* used a classical Cas9 protein that cuts both strands of DNA and induces DSBs, Chen *et al.* used a $Cas9^{D10A}$ protein which cuts only one strand of DNA and induces single-stranded breaks (SSB) [2, 3]. When two SSB are generated in a short distance of each other, HDR can be induced (for optimal effect, $Cas9^{D10A}$ nick sites should be separated by 37-68 bp) [18, 19]. In cells where the fusion gene partners are still located on their respective chromosomes (non-rearranged), only one SSB will be induced on the chromosome [2]. When only one SSB is generated by the $Cas9^{D10A}$ protein, the cut is repaired faithfully, as one SSB does not allow for the creation of the indels generated by NHEJ or gene addition generated by HDR [18]. Thus, only when the gene partners have rearranged to create a fusion gene will the SSBs be closely located together, allowing for the insertion of the suicide gene [2].

Comparison of strategies

While both of these potential cancer therapeutic strategies rely on cancer genome editing by targeting fusion genes, there are three differences between the strategies essential for further consideration of clinical use.

Firstly, the two strategies rely on different DNA repair mechanisms. In proliferating mammalian cells, the NHEJ pathway remains highly favoured over the HDR pathway; Mao *et al.* reported that for DSBs in human proliferating cells, 75% was repaired through NHEJ whilst the remaining 25% was repaired through HDR [20]. While Chen *et al*. rely on the less efficient HDR pathway, Martinez *et al*. rely on the more efficient NHEJ pathway, making the strategy of Martinez *et al*. more attractive. Indeed, for *in vitro* cancer cells, Chen *et al*. reported an editing efficiency of only 15.9% to 25.5%, while Martinez *et al*. reported 82.1% efficiency [2, 3]. However, it is still difficult to estimate how efficient either pathway might be in other types of cancer cells that harbour the fusion gene, as this is influenced by a variety of factors that are likely to differ between different cancers. For example, decreased levels of p53 in a cell, as often observed in cancer cells, are known to promote the HDR pathway [21]. Additional research to investigate the efficiencies of NHEJ and the HDR pathway in different types of cancer cells *in vivo* is necessary to provide a better estimate of the potential clinical effect of cancer genome editing.

Secondly, while the strategy of Chen *et al*. is breakpoint specific, the strategy of Martinez *et al*. has the potential to be more broadly applicable [2, 3]. While fusion genes with a certain set of gene partners can be recurrent, the exact points at which they are fused can be variable [3]. The DNA knicks should be relatively close together (37-68 bp) to induce HDR with a high efficiency using the $Cas9^{D10A}$ enzyme [19]. Thus, in the strategy of Chen *et al*., flanking the breakpoint with such a short distance in between means that the gRNAs will have to be highly specific to the exact point at which the gene partners are fused [2]. In contrast, the exact breakpoint is not a big concern in the strategy of Martinez *et al*., as long as the gene partners remain the same, the gRNAs can target any introns flanking the breakpoint [3]. Thus, this strategy allows for a standardisation of gRNAs for a fusion gene, as there is little concern about the exact location of the breakpoint, making it more attractive as a mass-produced therapeutic.

Lastly, the two strategies have a different range of types of fusion genes they can target. For the gene deletion strategy of Martinez *et al*., the fusion gene that is targeted is required to be driving oncogenesis [3]. The suicide gene strategy of Chen *et al*., on the other hand, only requires the presence of a fusion gene as an insertion point for the suicide gene [2]. For the strategy of Chen *et al*., the only requirement for the fusion gene is that it is relatively widespread among the cancer cells, while in the strategy of Martinez *et al*., the inhibition of the fusion gene itself must have an anti-cancer effect [2,

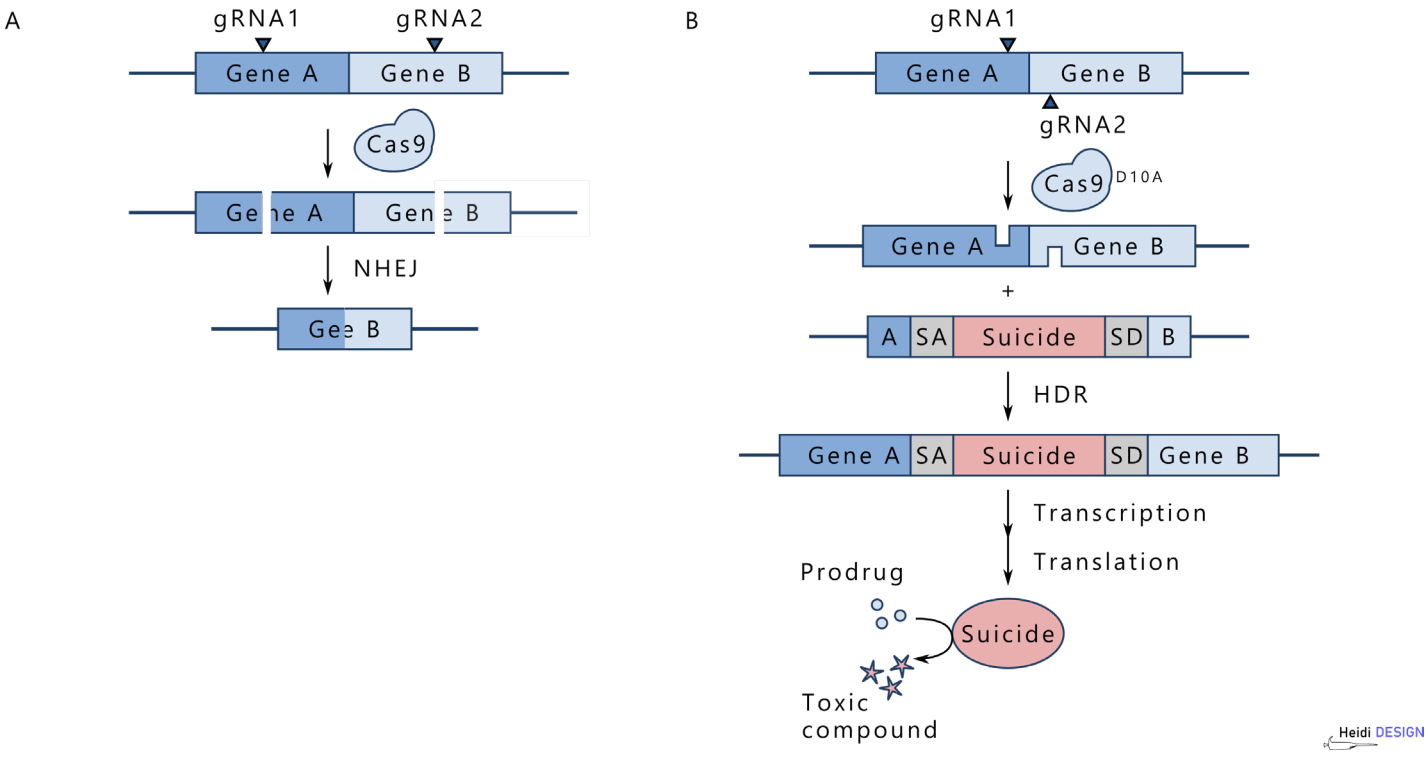

Figure 1: Cancer genome editing strategies targeting fusion genes

A. The gene deletion approach of Martinez et al.: two gRNAs are chosen, flanking the breakpoint of the fusion gene consisting of gene partners A and B. Cas9 induces two double-stranded breaks (DSBs) at the gRNA location, and the sequence in between the DSBs is removed by non-homologous end joining (NHEJ) [3].

B. The suicide gene approach of Chen et al.: two gRNAs are chosen closely, flanking the breakpoint of the fusion gene consisting of gene partners A and B. The $Cas9^{D10A}$ protein will cut, and homology-directed repair (HDR) will use the recombination donor to repair the cuts. The recombination donor consists of a suicide gene flanked by 1) a splice donor and a splice acceptor to allow for correct splicing and 2) sequences homologous to gene A and gene B to allow for activation of HDR. The transcript containing gene A, the suicide gene, and gene B is generated, and independent translation activation will produce the suicide protein. The suicide protein will convert a prodrug into a toxic compound, leading to cell death.

3]. Furthermore, inhibition of fusion genes is not limited by cancer genome editing; for example, small molecule inhibitors of fusion gene proteins involving tyrosine kinases are already in clinical use [15]. Thus, the gene deletion strategy would have to show strong advantages over these small molecule inhibitors for it to replace the current standard of care. Overall, the suicide gene deletion has a wider variety of potential fusion gene targets than the gene deletion strategy of Martinez *et al*. [3].

Altogether, the gene deletion strategy by Martinez *et al*. relies on the more efficient NHEJ repair mechanism and its gRNAs are more easily standardised for a specific fusion gene, while the suicide gene strategy by Chen *et al*. has a broader range of applicability of fusion genes [2, 3].

Challenges

Currently, CRISPR/Cas9 mediated genome editing of cancer still faces many challenges that must be overcome before it has a chance of reaching clinical use, which mainly centres around safety and efficacy. A great potential advantage of CRISPR/Cas9 cancer editing is that the therapy should be entirely specific for the cancer cells when it is correctly targeted to, for example, a fusion gene and, thus, might have limited side effects. However, CRISPR/Cas9 genome editing does come with other safety concerns, including the safety of the delivery system and potential off-target editing effects. Both cancer genome editing strategies used the adenovirus vector to deliver the gene-editing tools into the tumour cells; however, immune-related toxicities remain a concern for *in vivo* treatment with viral vectors [22]. Both approaches for cancer genome editing targeted fusion genes and found no significant effect of the treatment on cells that did not harbour the fusion gene. However, potential toxicity of the adenovirus vector and any off-target cutting effects should be closely monitored in future research [23, 24]. Furthermore, ethical and safety concerns remain, considering the potential of germline genome editing [25]. Were a CRISPR/Cas9 genome editing therapeutic delivered systemically, as one might imagine for tumours that are difficult to reach for direct injection, or in the case of treating metastasised cancer, any off-target cuts in germline cells could be inherited by the offspring of the patient.

The efficacy of a cancer genome editing strategy as a therapeutic is dependent on many variables. This includes the editing efficacy, which depends not only on the repair mechanism but also on the delivery strategy [26]. Interestingly, the injection of an adenovirus vector has been shown to activate an anti-tumour immune response that can affect even non-injected tumours, possibly increasing the effects of genome editing therapy [27]. Like most cancer therapies, it is possible that cancer genome editing will eventually lead to cancer cells resistant to the therapy, but this has not been well characterised yet and requires further research. Overall, additional investigation is necessary to evaluate the efficacy of cancer genome therapy as compared to current standards of care.

Conclusion

All in all, in the future, CRISPR/Cas9 genome editing might be a viable therapeutic for cancer treatment. Two proposed strategies that target fusion genes have indicated that CRISPR/Cas9 genome editing of tumour cells *in vivo* is both effective and safe [2, 3]. Whether either strategy will eventually be suitable for clinical use will depend on further research into their efficacy, safety, and comparison to current treatments. While both the gene deletion strategy and the suicide gene strategy showed strong anti-cancer effects, the gene deletion strategy might be more practical due to its reliance on the efficient NHEJ repair strategy and its potential for therapeutic standardisation for specific fusion genes [3]. However, for the gene deletion strategy to be effective, the targeted fusion gene would have to be a strong driver of oncogenesis, whereas for the suicide gene strategy merely a widespread fusion gene has the potential to be a target [2].

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MIND THE GAP:
FACING SEX BIAS IN BIOMEDICAL RESEARCH

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Over the years, an "androcentric" bias has dominated biomedical research with male subjects and animals being overrepresented in (pre)clinical studies [1]. Indeed, for decades, medical textbooks have defined the "70-kg man" as the default human model for studying human physiology [2]. As a consequence, women have been largely excluded from biomedical research over the years. There are, of course, many biological similarities between the two sexes that prompted this simplification. However, there are multiple layers of variation between males and females. Sex differences begin at the genetic level where a chromosomal composition of XX in females and XY in males orchestrates changes on the anatomical and physiological features of each sex [3]. Besides genetics, hormonal factors are also crucial to differentiate between males and females [4]. Such hormonal differences are responsible for the development and maturation of the reproductive system, as well as specific behavioural and cognitive traits in each sex [4]. On an organismal level, women generally have lower muscle mass, higher body fat mass, and lower blood pressure compared to men [3]. Collectively, genetic, endocrine, and physiological variation between the two sexes can affect their disease risks, illness patterns, symptoms, and even treatment efficiency.

History and state-of-the-art

The underrepresentation of women in biomedical studies has been present for a long time. "Hundred years ago, medicine was a men's domain. In discourse, the representation in research and textbooks was just men. This does not mean they all thought females were inferior, it was just an ignorance for the differences between the male and female body", says Sabine Oertelt-Prigione, professor of sex- and gender-sensitive medicine at the Radboudumc. "A huge pullback was the thalidomide crisis in the '70's, where babies were born with malformations after their mother had taken thalidomide during pregnancy. This led to a reaction by regulatory agencies that wanted to protect unborn babies, so they excluded women from clinical trials." In the '90's, researchers realised that sex differences have a larger impact on healthcare than previously thought [5, 6]. "What happened is that women were permitted to participate in clinical trials again, and we have seen an uptake ever since", notes Oertelt-Prigione. Yet, a 2019 analysis observed clear sex bias in clinical studies from Pubmed and Clinicaltrials.gov [7]. Strikingly, the analysis only found a small increase in the number of female participants over the period of 1966 until 2018 [7]. In another study, phase I trials were found to have more sex bias than phase II and III trials [6]. Thus, women are still underrepresented in early drug development, which could lead to sex differences being unnoticed until later stages of clinical development.

Sabine Oertelt-Prigione, MD, PhD, Radboudumc.

"20 to 25 years ago, people started looking at sex differences incidentally, but there was no systematic integration", says Oertelt-Prigione. "Considering that sex was perceived as an emancipatory political statement rather than a content analysis, when I tell people 'well, I work in sex- and gender-sensitive healthcare', they start telling me the number of female professors they have in their institution, which is very important but not the point." The last five years, things are coming together. First, there is an accumulation of knowledge that convinces more people to look into sex differences. Second, funding bodies mandate that researchers consider sex differences in their studies. The National Institutes of Health expects researchers to include sex as a variable in research design, analysis, and reporting [8]. If researchers do not incorporate sex in their study, they need to explain why not [8]. The Canadian Institute of Health has also commenced such regulations since 2010 and has become stricter over the years [9]. Likewise, the European Commission has mandated to include sex via the Horizon 2020 program [10]. "This does not mean everybody does it [integrating sex in biomedical research] systematically and perfectly, but it encourages researchers to think about whether their study has a sex dimension." Lastly, as Oertelt-Prigione explains, publishing about sex differences in journals and even high-profile journals is increasingly possible. "At this point, it becomes a

virtuous cycle where agencies want it, there is more knowledge, and you can get it published decently. So, the process is kind of encouraging itself."

Why is this an issue?

Sex differences are evident in many pathological conditions such as diabetes, Alzheimer's, and cardiovascular diseases [11]. For example, compared to men, women suffering from coronary artery disease are older and usually do not experience an obstruction in large blood vessels at the same rates [11, 12]. Differences in pathophysiology and symptomatology between the two sexes can complicate proper identification as well as treatment of major chronic diseases [11]. Thus, establishing sex-specific guidelines for the diagnosis and prevention of such diseases is urgent. Perhaps one of the most dangerous consequences of the exclusion of females from clinical trials involves sex-specific responses to therapy [13]. "We are giving a drug to somebody, and we would like to know whether it is as efficacious in females as in males, and, of course, if it shows the same side effects in both [sexes]", mentions Oertelt-Prigione. Not only therapy efficacy but also treatment-related side effects can be different in males and females prescribed the same medication [13]. The one-size-fits-all norm often applied in medicine has led to the overmedication of women and is associated with increased adverse drug reactions in women [14]. Oertelt-Prigione notes, "There are some cases where drug dosages have been adapted but were still as efficacious and without side effects. Notable examples are the use of certain sleep medications and even chemotherapeutics." A systematic analysis performed in 2016 reported that about half of the drugs (307), most frequently prescribed in the USA, are associated with sex-specific adverse reactions [15]. These differences are mainly attributed to sex-based differences in pharmacokinetics and pharmacodynamics [13]. Therefore, the exclusion of females and ignorance of sex-based differences in (pre)clinical research can have detrimental effects on women's health. Oertelt-Prigione concludes, "This is a field where we expect substantial progress in the next years and potentially even different types of medications for men and women. Coming to the future, we can adapt our therapies to tailor the medication to whomever the individual taking it is."

Sex-(in)sensitive research fields

The incorporation of sex differences is not equal among the various biomedical fields. "Cardiology is probably the most advanced field in sex-specific research", says Oertelt-Prigione. "Realising there was a higher incidence of heart attacks in women due to different symptomatology or simply because the physician did not think women were having heart attacks, highlighted the importance of sex-based differences in cardiology". Indeed, research into cardiovascular diseases in women has led to the establishment of sex-specific guidelines for the risk, diagnosis, and treatment of such diseases [16]. For instance, special protocols for pregnancy and menopausal related risks are now being recommended in cardiology practice [16]. Yet, systematic analysis of articles published between 1966 and 2018 revealed a clear underrepresentation of female participants [7]. A negative sex bias, indicating male overrepresentation, was observed in multiple research fields, including that of HIV/AIDS, kidney diseases, and even cardiovascular diseases [7]. Oertelt-Prigione discusses, "There are fields that are a bit more advanced and fields that are lagging a bit behind. I would say that my impression is that there are fields where we know a lot, and still, nothing is happening. It is also true that in many fields sex-disaggregated data is lacking and, therefore, larger meta-analyses are difficult to conduct. As a consequence, we do not have a high enough level of evidence to include this knowledge into clinical guidelines."

Tools to support sex-sensitive research

Despite the advances made in the last years, integrating sex-based differences is still limited. Oertelt-Prigione explains, "There is some ignorance or lack of information about how to conduct sex-based analyses. Not that much because of bad intentions, but because of a lack of knowledge. Besides, there are more practical considerations, for example, the statistical analysis and instruments you use in your study. All these make implementation difficult". There are many ways to stimulate and guide researchers to consider sex-based differences in their studies. Oertelt-Prigione discusses, "I think there are different steps and levels depending on where people are. Initially, what you want is to raise awareness that there is a problem. That is what I do in a way by giving talks to the community. The second level is providing researchers with tools and experts to accompany them." Recruitment, randomisation, data collection, and data analysis can be optimised to include sex-specific differences [17]. Educating the scientific staff is also important to promote their involvement into sex-sensitive research. Oertelt-Prigione notes, "If you have a faculty where there is a person who likes the topic or has this as a research subject, it will pop up during the teaching. But if you do not have somebody that embodies this, or carries the torch for this, it does not automatically happen. I really hope that in the next ten years we will move from these single experiences to more of an institutionalised process, where it is simply requested to perform sex-sensitive studies, and there is a trained faculty who can teach this." On a global level, multiple guidelines and online training tools have been developed to help researchers add a sex dimension in their research [8, 9].

The challenges of gender in the clinic

Alongside sex, gender differences can also influence biomedical research. Sex and gender are two similar but different concepts. Sex is considered a biological component, defined by the sex chromosomes, hormones, and anatomy [8, 9, 18]. Gender is a broader term and comprises the social, environmental, cultural, and behavioural factors and choices that influence a person's self-identity and health [9, 18]. This includes gender identity, gender norms, and gender relations [9, 18]. "Historically, the two concepts have been mixed. People thought sex was a dirty word, so they used gender instead." According to Oertelt-Prigione, researchers and physicians became more aware of the fact that the concepts are not the same in the last 15-20 years. "They both have an impact on healthcare but in a different way. Gender especially impacts access to healthcare, perception of health and disease, preventive behaviour, and the way diagnosis might be offered to you." Thus, including gender is as important as the inclusion of sex. Currently, this remains a challenge. Oertelt-Prigione explains, "Gender is a concept that comes from social studies. The studies in social sciences are very different from medical studies, so we need to make sure gender is measurable with instruments that work for healthcare studies. The current challenge is to make gender more usable in medical research. The knowledge is present, but the methods on how to incorporate it must be clearer and more distinct."

Initiatives at the Radboudumc

As a professor in sex- and gender-sensitive medicine, Oertelt-Prigione is working with many people at the Radboud University and Radboudumc. She mentions some initiatives within the Radboudumc to introduce sex and gender in biomedical sciences. "In this coming year, we are working on a project where we are trying to find out how to combine an innovative topic as sex- and gender-sensitive research with innovative teaching methods." For the future, she is hoping that teaching about sex and gender in biomedical research becomes natural. "By building collaborative projects with many different clinical disciplines, we hope to engage them so that they will mention it themselves when they are teaching." Besides, there

are other initiatives within the research institute. These range from performing research into the topic to scientific student lunches with PhD candidates and Research Integrity Rounds about the topic. "The most important aspect over time is that people know where to find you. Every once in a while, people contact me with their questions. That means people start taking up what we have said and translate it into the work they are doing."

Conclusion

From the earliest days of medicine, sex and gender differences have not been thoroughly considered in biomedical research. As a result, few and slow advances have marked sex and gender-sensitive research and medical practice. Nevertheless, the research landscape has evolved in the last years, encouraging sex- and gender-based analyses in biomedical studies. Oertelt-Prigione concludes, "The climate is definitely changing, and ten years ago things were much trickier. Not to say that everything is perfect, but we are in a moment where there is more awareness and people are more accepting and more interested. We will definitely see a lot of new things coming up in the future."

Acknowledgments

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OVERCOMING EPIGENETIC ROADBLOCKS

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Standfirst

Induced pluripotent stem cells (iPSCs) are invaluable tools both for research into pluripotency and development, as well as in the fields of regenerative and personalised medicine. They represent a unique resource, as they can be generated from a minimally invasive tissue sample obtained from an individual. However, much is still unclear about the molecular processes underlying this reprogramming and efficiencies remain low. The process of creating iPSCs requires resetting the epigenome of somatic cells toward a pluripotent chromatin state. A new chemical screen identifies epigenetic and signalling roadblocks for reprogramming of human somatic cells, with the inhibition of these roadblocks resulting in a more permissive epigenome for reprogramming. These findings shed light onto the complex epigenetic mechanisms underlying cellular reprogramming and are of high importance towards a more reliable production of iPSCs.

Accessible via: Raas, M.W.D., Zijlmans, D.W. & Marks, H. Overcoming epigenetic roadblocks. Nat Chem Biol (2021). https://doi.org/10.1038/s41589-020-0629-3 [1]

During development, pluripotent stem cells differentiate toward all specialised cell types of a mature organism [2]. A well-known metaphor of cellular differentiation is Waddington's epigenetic landscape. Essentially, embryonic stem cells start atop a steep hill early in development. Over the course of subsequent development, these cells roll down the hill while taking various routes, symbolising lineage commitments. In the end, the cells will have reached one of the many valleys surrounding the central mountain and are stuck here. In 2006, this dogma was challenged by showing that forced expression of a select combination of transcription factors induced somatic cells to revert to a pluripotent state, essentially representing a climb back up Waddington's epigenetic landscape (Figure 1) [3]. Cells reprogrammed in this way are known as induced pluripotent stem cells, and these have since been used to study the biological underpinnings of pluripotency and differentiation. Furthermore, their great potential in personalised and regenerative medicine is becoming increasingly clear (e.g. for patient-specific disease modelling and drug screening) [4]. However, reprogramming of somatic cells remains inefficient (between 0.1–3% of all cells successfully reprogram), and numerous important questions underlying the reprogramming process have remained unanswered, such as which features make donor cells refractory to reprogramming [5, 6]. In a recent study published in *Nature Chemical Biology*, Kim *et al.* show that chemical inhibition of epigenetic and signalling factors results in a chromatin state that is more permissive for reprogramming (Figure 1) [7]. This permissiveness not only improves reprogramming efficiencies but also broadens the scope of transcriptional regulators that are able to effectuate reprogramming.

Establishment and maintenance of cell identity are governed by epigenetic modifications. These include post-translational histone modifications and DNA methylation, which affect transcription factor binding and accessibility of genes, thereby regulating gene expression [8]. As such, cellular differentiation is associated with a widespread reorganisation of the epigenome. In view of the stability of the epigenome of somatic cells, the pre-existing epigenome is expected to pose a substantial barrier for cellular reprogramming toward pluripotency. To overcome this barrier, Kim *et al.* applied an extensive chemical screen targeting epigenetic and signalling pathways to identify the main roadblocks for inducing pluripotency of human fibroblasts [7]. Chemical inhibitors that drastically improved reprogramming efficiencies targeted five main pathways: (I) DOT1L-mediated mono- and demethylation of H3K79*; (II) LSD1-mediated H3K4 and H3K9 demethylation; (III) HDAC-mediated histone deacetylation; (IV) TGF-β signalling; and (V) DNMT-mediated DNA methylation—see figure legend for abbreviations. Notably, sequential administration of these inhibitors displayed a synergistic effect, revealing some of the complex hierarchical epigenetic dynamics that occur during reprogramming. In particular, inhibitors of DOT1L represented the most effective compounds in facilitating reprogramming. Besides an expected strong reduction in H3K79me1/2, inhibition of DOT1L consistently resulted in reduced global levels of H3K27me1/2. This is a remarkable observation, as there is no established crosstalk between H3K79me1/2 and Polycomb Repressive Complex 2, the catalytic protein complex mediating methylation of H3K27. Together, these results show that H3K79me1/2, possibly together with H3K27me1/2, represent a major barrier in reprogramming.

Despite the profound similarity on structural, spatiotemporal, and functional levels, only one of eight known OCT factors, namely OCT4, can complement the reprogramming factors SOX2, KLF4, and c-MYC (SKM) in facilitating reprogramming. Kim *et al.* attribute this to the differential genomic binding between OCT4 and the other OCT proteins, including OCT4-exclusive binding at the enhancers of pluripotency factors [7]. Interestingly, the more permissive epigenomic state resulting from DOT1L inhibition allowed for the substitution of OCT4 in the reprogramming cocktail by other OCT factors. The authors hypothesise that this might be facilitated by increased binding of these OCT proteins at canonical OCT4 genomic binding sites, including at the enhancers for important pluripotency-associated genes like *NANOG* and *OCT4*. These enhancers likely become accessible for binding of these OCT proteins due to loss of H3K27me2, as H3K27me2 keeps enhancers poised [9]. Additional factors that were found to be able to substitute OCT4 during reprogramming from a

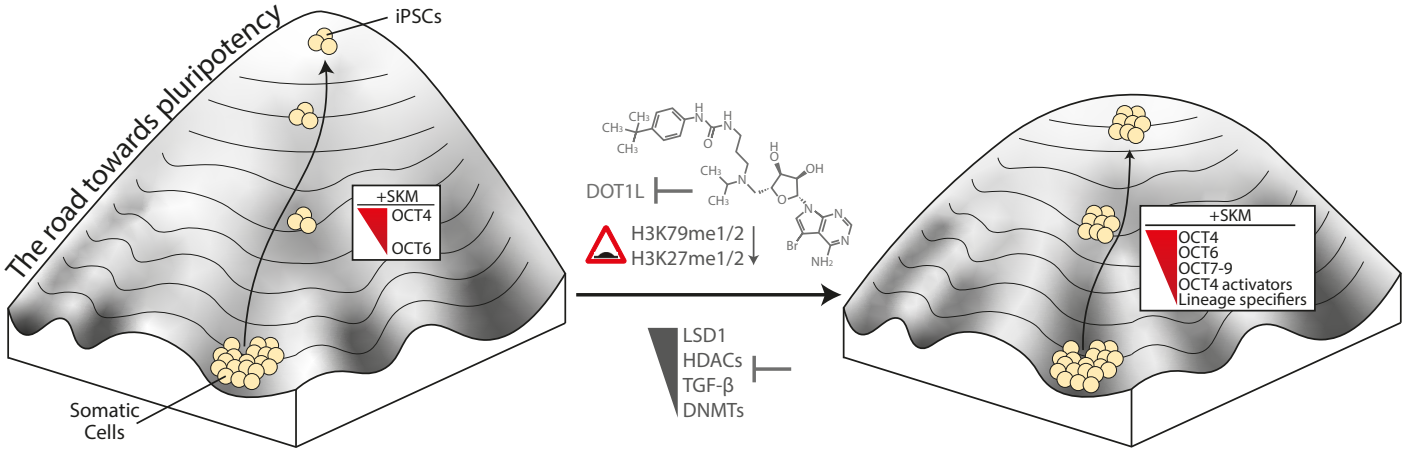

Figure 1: Targeted chemical inhibition alters the epigenome of somatic cells toward a more permissive state for reprogramming.
In these epigenetic landscapes of Waddington, the slopes represent barriers faced during the reprogramming of somatic cells toward induced pluripotent stem cells (iPSCs). Following chemical inhibition of key epigenetic and signalling pathways, major epigenetic roadblocks (including H3K79me1/2 and H3K27me1/2 by inhibition of DOT1L) are removed. As a result, the landscape adopts a more permissive state in which barriers for reprogramming are lowered. Consequently, the reprogramming efficiency improves, while also allowing identification of low-competent reprogramming factors. DOT1L, disruptor of telomeric silencing 1-like; LSD1, lysine-specific histone demethylase 1; HDAC, histone deacetylase; TGF-β, transforming growth factor-beta; DNMT, DNA methyltransferase; OCT, octamer-binding transcription factor; SKM, the reprogramming factors SOX2 (Sex determining region Y box 2), KLF4 (Kruppel-like factor 4) and c-MYC.

permissive epigenomic state include the lineage specifying transcription factors PAX4, FOXA2, and SIX3, confirming previous reports that these types of proteins can fulfil unexpected roles in reprogramming [10]. Together, these results provide exciting new insights into the mechanisms of reprogramming by showing that pre-existing epigenetic barriers such as post-translational histone modifications can limit the potential of such factors in inducing reprogramming.

In their article, Kim *et al.* provide important tools to improve the efficiency of reprogramming substantially [7]. However, low efficiencies remain a bottleneck. Therefore, an outstanding question is whether screening a broader range of (combinations of) inhibitors targeting epigenetic enzymes will reveal efficient chemical cocktails for reprogramming. With regard to the cells used, Kim *et al.* provide a proof of principle for their reprogramming screens in human fibroblasts [7]. Another important future question concerns whether there is a specificity of the epigenetic inhibitors with regard to cell type, and, if so, what are the associated epigenomic barriers for reprogramming of other types of donor cells. More mechanistically, the observations in the current study lay down the groundworks for in-depth analysis of the interactions between pre-existing epigenomes of donor cells and the transcription factors responsible for the activation of the pluripotency networks during reprogramming.

In conclusion, the pioneering study by Kim *et al.* provides invaluable insights into epigenetic and signalling roadblocks encountered during reprogramming [7]. This will pave the way toward more efficient reprogramming methods for human somatic cells, possibly by chemical compounds only, as has been demonstrated for mouse somatic cells [11].

**These abbreviations refer to specific histone posttranslational modifications and should be read as such: H3 refers to the histone H3 protein (which is one of the subunits that make up the histone octamer at the center of nucleosomes), K79 refers to the lysine residue (K) at the 79th amino acid position of the histone protein, and any further notations refer to a functional group that is covalently bound to the residue (i.e. me1 and me2 for mono- and dimethylation, yielding H3K79me1/2).*

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IMMORTALITY: A BLESSING OR A CURSE?

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The search for immortality has interested people for many centuries, with a great example being the famous painting 'The Fountain of Youth' created by Lucas Cranach in 1546 (Figure 1) [1]. This painting depicts older women who can barely walk, diving into a magical fountain, and emerging as younger and rid of their age-related defects. Nowadays, scientists are getting closer to discovering this magical fountain by exploring anti-ageing treatments that might increase your lifespan. But as we are so close to finding that recipe, would you like to slow down this process? Or would you rather dive into 'The Fountain of Youth' and meet immortality?

The road will end, prepare to die

Before we find the solution to ageing, we first have to understand why we become old and eventually die. All of us have 23 pairs of DNA, called chromosomes, in each cell of our body [2]. Each chromosome has special caps at its ends, the telomeres, which can protect the chromosome from damage [3]. When a cell divides during your life, the DNA inside the cell is copied to a new cell [2]. However, not all genetic material can be copied, and the chromosomes in the cell start becoming shorter [4]. The genetic material only becomes shorter at its ends, initially affecting the size of telomeres but eventually affecting essential DNA sequences as well [4]. The cell is not stupid. When its chromosomes get shorter and shorter, the cell will randomly decide not to divide anymore in order not to be destroyed, a concept known as cellular senescence [5]. And this is how ageing occurs [6]. Imagine ageing as a signal that turns healthy people into zombies. Zombies will stop functioning normally and will start causing various problems to the surrounding environment. They will chase surviving people and bite them to turn them into zombies as well. The more zombies are present in town, the bigger the damage and the faster the whole town will disappear. In this example, the town represents the human body, with zombies representing the non-dividing 'old' cells of our body that will chase and destroy the 'survivor' healthy cells. These 'zombie cells' are found to be responsible for ageing and will internally program us to die.

Anti-ageing drugs on Amazon

However, ageing itself is not the biggest problem, rather age-related diseases which can make your road to death even shorter. Scientists around the world are trying to solve the puzzle of ageing to avoid this decline. They try to find cures that aim specifically to fight the 'zombie cells' in our body or even keep telomeres longer so they can still protect the cell from damage. The battle against 'zombie cells' can be easily reinforced by 'superfoods' [7]. Some of these nutritional heroes are hidden in your food, such as Vitamin A or Vitamin C, which can save your body by scavenging the bad cells. Of course, if you eat all your vegetables during your dinner or a lot of fruit during the day, it does not mean that you will suddenly become ten years younger. However, it does mean that you could buy for quite some time until you reach your final destination. Another way of battling ageing is more complicated since you also have to use some drugs. There are several drugs on the market, even found on Amazon, that claim to keep your chromosomes long and, therefore, keep you young. These drugs are based on a molecule found in the Chinese herb plant *Astragalus membranaceus* [8]. When you orally ingest the drug, it circulates in your blood and eventually enters the old 'zombie cells' of your body. In there, it wakes up the guy who is responsible for making telomeres, the telomerase enzyme. Telomerase then starts working to make chromosomes longer and cells healthy again. However, such treatments might be too good to be true.

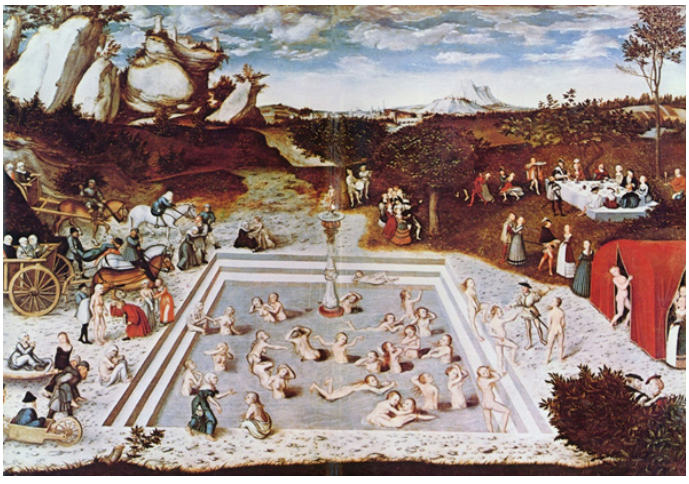

Figure 1: 'The Fountain of Youth' painted by Lucas Cranach in 1546

Warning, danger zone!

If you examine the painting of 'The Fountain of Youth' (Figure 1), you will notice a man, dressed in red and holding a book, trying to warn a lady before she jumps into the magic water. He might be a doctor or a crazy scientist. Contemporary scientists are actually trying to do the same. They warn us that all these anti-ageing drugs are not tested for long-term effects and might even have many dangerous side effects. However, some companies want to benefit from the attractive idea of anti-ageing and increase their profits by bringing these products to the market. But what do laypeople think about living in an immortal world? Journalists of the journal 'New Scientist' were curious enough to survey more than 2,000 British people on the topic of longevity [9]. The outcome was quite surprising since only 20% were positive towards this concept. Why would modern-day people 'reject' immortality? As it turns out, living forever hides many dangers.

Listen to the experts

"A lot of the value of life is drawn from its finite character. If you could truly always postpone everything until tomorrow, will you ever do things?" says Bart Penders, associate professor from the Department

of Health Ethics & Society at Maastricht University. "If we achieve immortality, the view of life and what life is will change. Everything and every personal relationship will get senseless", adds Jos Kole, assistant professor from the Department of IQ Healthcare Ethics at Radboudumc. Imagine a world in which everybody lives forever, where you have the same conversation with the same neighbours, every single morning, for your whole infinite life. Such a life is not so interesting anymore. "But of course, if everybody lives forever, then practical problems will arise too", warns Kole. If nobody dies, the Earth will run out of space and resources to sustain all human life, and eventually we would have to settle ourselves on a different planet. In addition, critical questions about the organisation of society would arise. Kole explains, "If we find the cure to ageing, who will it be available for? Who will decide who gets the treatment? How much more expensive will healthcare become?".

After what has been said so far, you might think that we are coming closer to finding the real 'Fountain of Youth'. However, with the oldest person alive today being 118 years old, we still have a long way to go until we can truly escape ageing. "Extending life is feasible, we are doing it now, but eternal life is still science fiction", concludes Kole. For now, enjoy the journey as long as it lasts.

Acknowledgements

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EXAM QUESTIONS

As RAMS aims to enlighten both students and professionals, we would like to present you two exam questions. Find out if you can remember what you have learned during your bachelor's!

We challenge you!

Question 1

During the acute phase reaction of an infection, the serum concentration of C-reactive protein and other so-called "acute phase" proteins increases. What is the stimulus for the increased production of acute-phase proteins?

A. Cytokines released by macrophages
B. Expression of integrins and selectins on the endothelium
C. Synthesis of reaction oxygen species in mitochondria

(Topic from Q2 The Immune system, 2019)

Question 2

Antagonistic pleiotropy is one of the mechanisms of ageing. An illustrative example of antagonistic pleiotropy is that:

A. APO lipoprotein allele epsilon 4 is necessary for fat metabolism at a younger age, whereas it increases the rate of developing Alzheimer's disease at an older age.
B. Patients develop a productive cough during pneumonia, which can cause a delirium in the eldery.
C. Hypertension forms a risk factor for heart- and vessel disease, but hypertension also supports the ongoing vascularisation of the brain at old age.

(Topic from Q2 MGZ Aging, 2019)

The answers to these questions can be found on page 29 in this journal.

ANIMAL RESEARCH IN MICE:
HOW WELL DO STUDIES IN MICE TRANSLATE TO HUMANS REGARDING VACCINE TESTING?

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Abstract

With companies filing new applications for a SARS-CoV-2 vaccine in record time, the public opinion regarding the safety and efficacy of these vaccines has become even more polarised. While the development time was too short to observe long-term effects in both pre-clinical and clinical studies, as of December 31st, 2020, some producers have been granted green light to start distributing their vaccines in various countries. Most of the animal data generated for the safety and efficacy of a novel vaccine is generated from mice. However, how well does the immune system of a mouse match the immune system of a human? How indicative are the results of mouse studies for human subjects (translatability)? This article aims to explore the key differences between humans and mice with regard to their immune systems, how this affects translatability, and how to further refine animal testing. The mural immune system shows functional similarities to the human immune system, with a few key differences in physiology, pathogen recognition, and antigen presentation. Nevertheless, the standard mouse model offers great opportunities to test mechanistic immunological hypotheses. Other mouse models, such as the "dirty" mouse model, commercially available transgenic mice, or "humanised" mouse models, may offer additional external validity, depending on the nature of the research.

In the light of the ongoing pandemic of the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), various medicinal developers such as Pfizer, Sinovac, and AstraZeneca have succeeded in creating a new vaccine in record time. As of December 31st, 2020, a total of seven unique vaccines have been granted (preliminary) approval for use against SARS-CoV-2 in various countries around the world. Another 55 candidates are currently in development or awaiting approval, according to the Regulatory Affairs Professionals Society (RAPS) vaccine tracker. One of the most remarkable aspects of the scientific response to the SARS-CoV-2 pandemic is the short amount of time it took from the identification of the first case in December 2019 to the (preliminary) approval of the first SARS-CoV-2 vaccine in December 2020. Following a successful phase II/III-type study, the Pfizer vaccine candidate, BNT162b2, was administered outside of a study setting for the first time in the United Kingdom on December 2nd, 2020 [1].

In a normal situation, exploratory work in animals can already take years [2]. Next to this, all phases of clinical testing, conventionally, last at least two years each, as patient recruitment, licence application, and the actual testing can all take some time [2]. The full process of discovering, manufacturing, testing, and approving a novel vaccine in a normal situation takes approximately 10-15 years [3, 4]. Animal testing is a standard procedure within the exploration of novel compounds and therapies. By exposing animals to a novel compound or therapy, the feasibility of developing the compound or therapy can be further assessed. However, there is a discourse among scientists regarding the relevance of animal testing [5-7]. In immunology, new vaccines are generally first tested in a rodent species. If the new product appears safe and effective in rodents, non-rodent species, such as sheep, goats, pigs, or non-human primates, are used to confirm the results from the rodent study. If the vaccine still proves to be efficacious and safe in the follow-up study, the product-candidate can move up to human clinical testing if the manufacturer deems it feasible after appropriate dose adjustments [4].

Nevertheless, a few SARS-CoV-2 vaccine candidates moved straight from *in vitro* to *in vivo* testing in non-human primates, skipping the rodent testing [8]. Data generated in rodent studies from previously generated SARS-CoV vaccine or MERS-CoV vaccine research was used to approximate how a SARS-CoV-2 vaccine should work [9, 10]. How well do rodent trials indicate the human effects of the same vaccine? This narrative review aims to highlight and summarise key differences between mice and humans regarding immune responses and the effects these differences may have on the translatability of mice data to humans. Providing a summary of the key differences between humans and mice regarding the immune system will aid in optimising immunological animal experiments.

The primary goal of a vaccine

Current vaccines often have the primary goal of establishing long-lasting cellular and humoral immunity to a certain infectious disease [11]. After vaccination, an immunised individual will have a circulating concentration of specific antibodies against the pathogen. These antibodies will help to neutralise viruses directly or mark pathogens that can then be neutralised by other immune cells. If a pathogen still were to infect cells after vaccination, the built-up cellular immunity helps to kill the infected cells [11].

The development, dosing regimen, and success rate of the vaccine depend on the type of vaccine. Historically, there are four main types of vaccines: live-attenuated vaccines; inactivated vaccines; subunit, recombinant, polysaccharide, or conjugate vaccines; and toxoid vaccines [12]. While live-attenuated vaccines can provide lifelong immunisation after one or two doses, these vaccines can usually only be given safely to relatively healthy, young, and immunocompetent persons [11-13]. The other types can be given to a wider population but will require booster vaccines later in life to maintain immunity [11-13]. In the past ten years, other types of vaccines, such as naked DNA vaccines or mRNA vaccines, have entered the market as well [10, 14]. mRNA vaccines appear to be useful in a wide array of vaccine targets [14].

Human immunisation pathways

In humans, the construction of life-long immunity to a new pathogen is an intricate process involving the innate immune system and the adaptive immune system. The innate immune system is non-specific and has little to no memory, while the adaptive immune system is specific and can offer life-long immunity [15, 16]. The innate immune system consists of the following factors, that are generally aimed at preventing infection: physical barriers, such as the skin; secretory defences, such as gastric acid; and non-specific cellular responses, such as macrophages [15, 16]. The adaptive immune system consists of cellular immunity, regulated by T-cells, and humoral immunity, regulated by B-cells [15, 16]. The innate immune system also plays a role in the activation of both cellular- and humoral immunity [11].

Cellular immunity

The process of generating cellular immunity starts with an immune cell, such as a monocyte, macrophage, or dendritic cell (DC), finding a pathogen in their environment (i.e. blood, tissue, and tissue, respectively) and engulfing it (Figure 1, panel 2) [15, 16]. These immune cells use a group of pattern recognising receptors to sense and recognise pathogen-associated molecular patterns (PAMPs) [11, 15-17]. These pattern recognising receptors are divided into subsets, all recognising a unique (set of) pattern(s) [15-17]. A specific example is the Toll-like receptor (TLR) 3, which recognises double-stranded RNA [15, 17]. These subsets combined cover the identification of a wide variety of pathogens. The PAMPs can be (part of) the pathogen or can be encountered alone [15].

Once a pathogen is "recognised", the cell that engulfed the PAMP or the whole pathogen will start to secrete large amounts of pro-inflammatory cytokines [15, 16]. In addition, the immune cell will start the process of antigen presentation (AP) to activate the adaptive immune system (Figure 1, panel 3-4) [15, 16]. Although monocytes, macrophages, and DCs can all be antigen-presenting cells (APCs), DCs are considered the most competent APCs to initiate a T-cell response [15, 16]. The DC will mature and migrate to a local lymph node, where it will present a small peptide from its engulfed pathogen to a naive T-cell through the process of AP [15, 16].

During AP, the mature DC must present three signals to naive T-cells to activate the T-cell [10, 15, 16]. The first signal consists of the presentation of fragments of the pathogen in the human leukocyte antigen (MHC/HLA) molecules on the DC's surface to the T-cell receptor [15, 16]. Secondly, the DC must provide co-stimulation through the

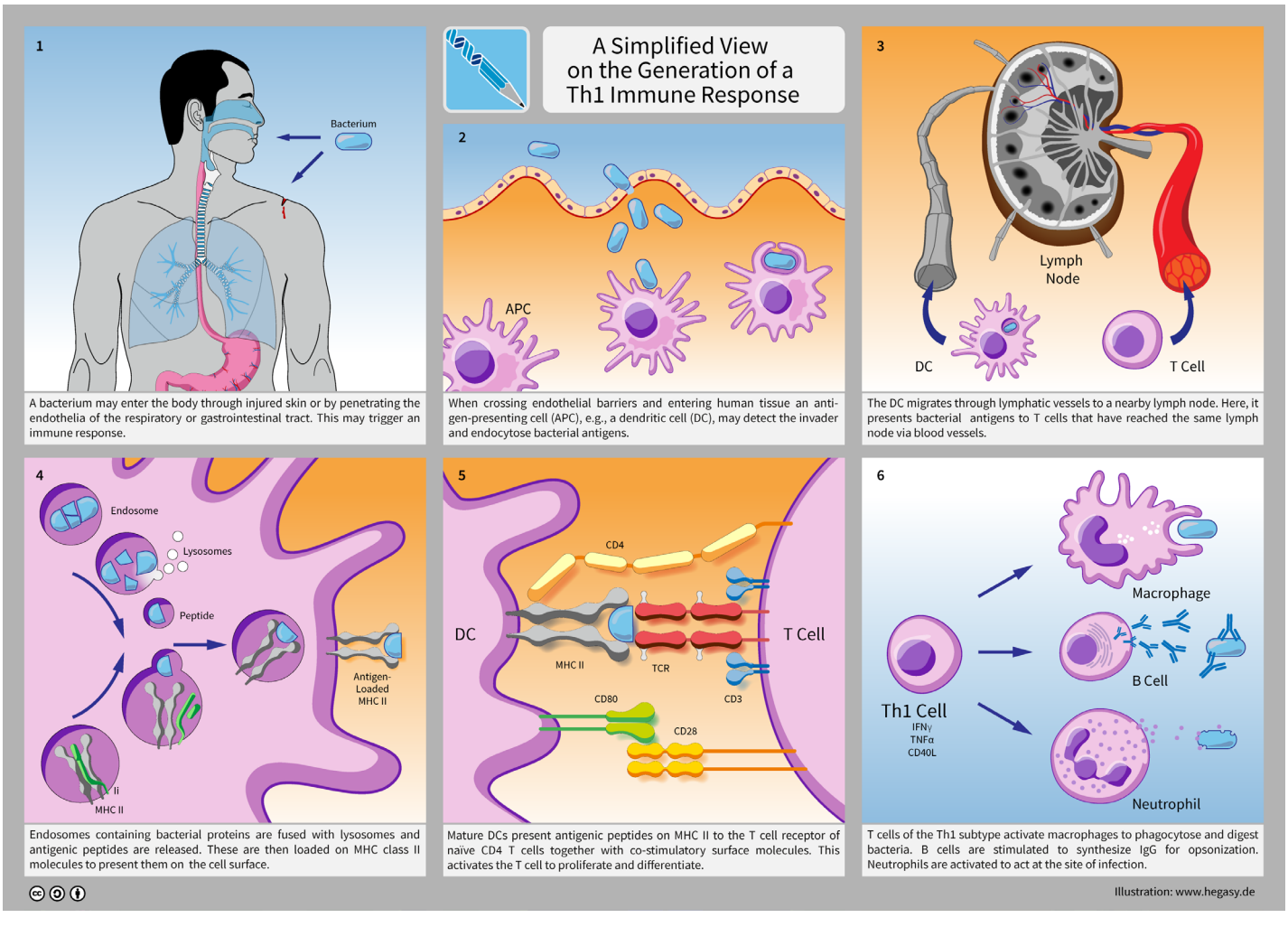

Figure 1: A simplified example of a Th1 adaptive immune response.

Th1 = T-helper1; MHC-II = major histocompatibility complex II; CD = cluster of differentiation; TCR = T-cell receptor, IFNγ = interferon γ; TNFα = tumour necrosis factor α. Illustration: www.hegasy.de

interaction of its ligands CD80 and CD86 with receptor CD28 on the T-cells [15, 16]. The third requirement is the secretion of stimulatory cytokines, such as IL-12, by the DC [15, 16]. If all three signals are present, an intracellular cascade is activated in the T-cell, resulting in a pro-inflammatory gene response [16]. The outcome of this process is T-cell activation, cell proliferation, and the polarisation to one of the following three types of T-cells: CD4+ pro-inflammatory T-helper (Th) cells, CD4+ regulatory T-cells, or CD8+ cytotoxic T-cells (Figure 1, panel 5) [11, 15, 16].

The CD4+ pro-inflammatory Th cells can be categorised further, depending on which type of adaptive immunity they provide [15, 16]. Th1 cells produce interferon-γ and tumour necrosis factor-α and protect from intracellular pathogens; Th2 cells produce IL-4, IL-5 and IL-13 and provide protection from extracellular pathogens; Th17 cells produce IL-17 and provide protection from fungi (Figure 1, panel 6) [15, 16]. CD4+ regulatory T-cells help dampen the immune response to prevent autoimmunity. Finally, CD8+ cytotoxic T-cells protect against viruses by killing infected cells after presenting a viral peptide on their HLA-I complex. Vaccination can help cellular immunity by establishing a T-cell response to a specific antigen, leading to an effective clean-up the next time the antigen is encountered [11].

Humoral immunity

The process of humoral immunity centers around the creation of antibodies against the pathogen. The process starts when a B-cell finds a PAMP and internalises the PAMP via endocytosis [15]. The internalised PAMP is then processed in a relatively similar manner as in other APCs. B-cells present the antigen on their HLA-II complex and wait for a Th cell to bind to the complex [15]. This linkage will trigger cytokine production by the Th cell, which will, in turn, cause B-cell hyperproliferation, formation of plasma cells, and the formation of memory B-cells (Figure 1, panel 6) [15]. The plasma cells will start to produce antibodies specific to the pathogen and release them into the surrounding tissue, while the memory cells will go dormant and support a rapid humoral response the next time the antigen is encountered [15, 16]. The human antibodies can be divided into the following categories: IgA1, IgA2, IgD, IgE, IgG1, IgG2, IgG3, IgG4, and IgM [15, 16, 18]. Each category is found in a specific niche and has a different role in the immune response [15]. For example, IgG antibodies can pass the placenta and help construct foetal immunity, while IgA antibodies are found in mucous membranes and help prevent the pathogen from infecting the individual before physical barriers are crossed [15]. Antibodies provide immunity by binding to pathogens, which neutralises them directly or makes it easier for other parts of the immune system to get rid of the pathogen [12]. Vaccination can help humoral immunity by establishing a basal antibody level and a fast antibody response to a specific antigen [11].

Non-human immunisation

The general pathway of immunisation is well conserved in jawed vertebrates [19]. However, there are small differences between vertebrate species regarding the exact cells, receptors, or signalling molecules involved in acquiring immunity. Even though the exact molecules may vary, characterising the relevance of the differences is challenging since both the human- and the mural immune systems do provide ample protection against pathogens. The awareness of the key differences between the human and mural immune systems outlined in this article can aid researchers in interpreting the results from their animal models.

Key differences

The major risk of testing a vaccine in a different animal than the intended target (i.e., humans) is that the actual pathogen may have a different method of action in the test animal [20, 21][13]. There is no benefit to using this animal model if the test animal cannot be infected by a particular pathogen, cannot form an immune reaction against it, or has a different mode of pathogenesis [20]. Recent research has made use of "humanised" mouse models to tackle this problem, which in the context of immune research means that immunocompromised or immunodeficient mice were injected with human immune factors, such as human immune cells or antibodies [20-22]. Another method is to genetically modify the animals to include human proteins [20-22]. These models appear to work well and may yield better predictions for the effects in humans [20-22]. In the case of SARS-CoV-2, a wild-type mouse model could not be infected by SARS-CoV-2. Instead, a transgenic mouse model and a mouse model engrafted with human angiotensin-converting enzyme 2 (ACE2) receptor had to be created to obtain mouse data about SARS-CoV-2. These transgenic models seem to mimic the human situation [23, 24].

Nevertheless, there are a few differences between mice and humans that change how an immune response is initiated. For example, mice express CD28 on all CD4+ and CD8+ T-cells. In humans, only 80% of CD4+ and 50% of CD8+ cells express CD28 on their cell membrane. This discrepancy means that as long as stimulatory cytokines are present, any mature T-cell can be activated by an APC in mice, while only 80% of CD4+ and 50% of CD8+ of T-cells can be activated by APCs in humans. As a result, mural vaccination may be more effective than human vaccination, both in acquiring immunity and in inducing an immune response after subsequent exposure to the pathogen.

Secondly, mice express no MHC-II on their endothelial cells [18]. Human endothelial cells, on the other hand, express MHC-II on their cell surface and, in this way, function as APCs [18]. This contrast means that therapies may have additional effects in humans compared to mice. In the case of vaccination, this means that additional routes of administration of the vaccine may also be viable in humans.

Furthermore, mice express caspase 8 but not caspase 10 [18]. Caspase 8 and 10 are downstream proteins of cell death receptors [18, 25]. Caspase 10 plays an essential role in the programmed cell death of T-cells in humans [18, 25, 26]. Mice that do not express caspase 8 are not viable, while humans without functional caspase 8 are immunocompromised [18, 26]. As these rodents lack caspase 10, mouse T-cells do not have programmed cell death [18]. Thus, using mice as a model for establishing T-cell immunity may overestimate the effect of a vaccine.

Additionally, there are differences in the signalling of TLRs between mice and humans. It is unclear what kind of effect this has on vaccine research translatability as a whole [18]. The most essential observed differences consist of the following: mural TLR5 is more sensitive to detecting bacterium-derived flagellin [27]; mice express TLR11, which detects uropathogenic bacteria—humans do not express this TLR [27]; mice do not express TLR10 in their innate immune system [28]; and TLR8 appears to have no function, or at least a different function in mice [29]. Due to these differences, mice can be considered better at recognising bacterium-derived flagellin and uropathogenic bacteria, while humans have the edge in identifying single-strand RNA viruses and keeping their innate immune response to an appropriate level [17, 30]. Thus, different vaccination strategies may have different effects in humans compared to mice.
Finally, there are morphological and physiological differences bet-

ween mice and humans. For example, AP can take place much faster in mice, as they have a much smaller body than humans, meaning the APCs have to travel shorter distances [18]. In an extreme example, AP could take place after only 20 minutes in mice, whereas it can take up to 12 hours in humans, resulting in a different time frame regarding the immune response [18]. Comparably, the human immune system has to maintain a much broader spectrum of antigen-specific T- and B-cells for a much longer time; up to 80 years for life-long protection [18]. Next to this, mice and humans have a different maturation timeframe of the immune system, which means that the results gathered from young mice are poorly translated to young humans [31].

It is also important to note that the animals used in laboratories are generally kept in a clean area [32, 33]. As a consequence, most lab mice will encounter fewer pathogens than in the wild, making their immune responses much weaker compared to their wild counterparts, also called "dirty" mice [32, 33]. This difference may lead to an underestimation of the effect in humans. Recent publications suggest that these "dirty" mouse models would be better models for immunology in adult humans [20, 33-35]. However, the "dirty" mouse model may also have negative sides. Experiments using "dirty" mice will have a higher in-experiment variability compared to the standard inbred strains of mice. While inbred mice are (almost) genetically identical to one another, each wild mouse can have major genetic differences compared to another wild mouse [32, 33, 35]. Wild mice also do not have identical exposures to previous pathogens compared to one another, making standardising the immune response hard, or even impossible, in the "dirty" mice [33]. The possible translational gain of using the "dirty" mouse model must be weighed against the likely increase of the number of animals needed to combat the in-experiment variability.

Discussion

Overall, mice show similarities with humans regarding the immune response and the specific immune pathways [18]. Most of the components of the immune system are highly conserved between different mammalian species. However, researching and acknowledging the differences between mice and humans is key to progress within animal science [4, 11, 32]. A higher translatability between mice and humans will lead to a higher success rate of novel treatments in human trials and reduce the number of animal experiments [20, 36]. Mice are the go-to choice of animal researchers to investigate the effects and safety of a new vaccine [35]. Modifications to the traditional mouse model, such as the use of "dirty" mice or the use of transgenic mice, provide additional opportunities to increase translatability [20, 33-35]. During the development of a vaccine to SARS-CoV-2, limited mouse data was generated before the producing companies decided to move to trials in larger animals or run early human clinical trials concomitantly to animal testing [8]. Time will tell if rodent data will still be considered necessary in future vaccine development.

Previous research, such as a 2004 review by Mestas and Hughes, reports a comprehensive list of differences between mice and humans [18]. These lists are extensive, and at this stage, it is not clear which differences between mice and humans are relevant for a specific outcome, such as successfully acquiring life-long immunity. As a consequence, researchers may not employ the optimal animal model in preclinical studies or the optimal experimental design in preclinical and clinical studies [21]. Mouse models offer great opportunities for exact mechanistic research questions, as models can be modified with a genetic alteration [21].

However, some researchers have been steering away from animal testing. Minimisation of animal use in medical research seems to be the course for the future [37]. Slowly, new methods are being explored to replace, or at least reduce, the use of animals in research [37]. Organoids and organ-on-a-chip methods can be used to model organ responses in human tissues but cannot be used to assess systemic outcomes [23]. *In silico* modelling is a more established method of gaining crucial information about the mode of action of drugs. It can be used to predict treatment outcomes through pharmacogenetics or to predict the behaviour of a drug through physiology-based pharmacokinetic modelling [37, 38]. These *in silico* models are already in use in the field of vaccinology [39, 40]. Groups such as SYRCLE have also been advocating for an increase in the use of systematic reviews of animal-based research to reuse already available data and thereby decrease animal suffering. For future vaccine trials for new diseases, the immunological pathway can be tested *in vitro* or *in silico*. As long as animal studies are necessary, besides previously mentioned alternatives, mice should be used to investigate the safety and efficacy of vaccines as long as they resemble the human immune system sufficiently; therefore, animal model selection and experimental design should be based on systematic reviews.

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THOUGHTS OF A MEDICAL STUDENT: "HOW DO YOU NOT TREAT A PATIENT?"*

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Guus Brand is a medical student at the Radboud University in Nijmegen and our standard columnist. He writes about any pecularities or striking events he encounters during his study program. This column will be about the difficult end-of-life decisions that physicians have to make during their career.

For Christmas, I received the book "Die ene patient" by science journalist Ellen de Visser. It is a bundle of her weekly columns in De Volkskrant, in which she interviews physicians about that one patient who changed their view on their profession. Many of these stories are hopeful and end in a full recovery, but too many end with palliative sedation, euthanasia, or ceasing of treatment. As a medical student and aspiring doctor, these latter stories in particular grip me, as I recognise that I do not feel prepared to make the existential choices these brave doctors, nurses, and other healthcare workers were able to make. I wonder, how does one choose to cease treatment, in consultation with the patient, and let a life slip away? How do you not treat a patient?

Do not get me wrong, I am well aware of the theoretics of when I should choose, in consultation with the patient, to stop the treatment a patient and let them pass away. I am informed of the possibilities and impossibilities of treatment in the final stage of life and the physiology of dying gracefully and peacefully. It is not the medical and theoretical aspect that is daunting to me, but the actual decision is. Many medical students, including myself, are not yet set on how to deal with our own mortality. However, as young professionals we are expected to be able to make decisions that might, both actively and passively, end a life. This is one of the aspects of the job that frightens me the most.

In my opinion there should be more space for learning how to handle such existential questions, particularly in the bachelor curriculum. It has come to my attention that several medical interns have sought spiritual guidance with the local pastor of the student's church, mainly to talk about the final stages of life. Even though it is fantastic that the church offers this support and guidance, it should not be necessary. As far as I am concerned, this kind of existential guidance should be an official part of the curriculum. It should be a full course, with European Credits as a reward if needed. Instead of conventional teaching and examination about evidence-based knowledge on existentialism and death, I believe that there should be room to talk to one another. I want to encourage you to discuss with each other and those with personal experience regarding what it is like to administer the final dose of morphine. What does it feel like to cease treatment in consultation with a patient, and how does one cope with doing so? Talk to terminally ill patients about how they feel about their final stages of life. Personally, I feel that such an addition to the curriculum would better prepare us for the reality of what it is like not to treat someone, and, therefore, what it is like to bring a life to an end.

Additional reading sources

Are you intrigued by palliative care after reading this column? One of the books that the author recommends is the novel "Being mortal" by Atul Gawande, an American surgeon. Additionally, you can have a look at the following articles selected by RAMS:

- Brigton, L., Bristowe, K. Communication in palliative care: talking about the end of life, before the end of life. *Postgrad Med J* **92**, 466-470 (2016).
- Arantzamendi, M., Belar, A., Payne, S., Radbruch, L., Hasselaar, J, Centeno, C. Clinical aspects of palliative Sedation in Prospective Studies. A Systematic review. *Journal of pain and symptom management* **14**, 1-25 (2020).
- Hui, D., Hannon, B., Zimermann, C., Bruera. E. Improving patient and caregiver outcomes in oncology: Team-based, timely, and targeted palliative care. *CA Cancer J Clin* **68**, 256-276 (2018).
- Galekop, M., Van Dijk, H., Exel, J., Cramm, J. Views of professionals and volunteers in palliative care on patient-centred care: a Q-methodology study in the Netherlands. *BMC Palliat Care* **18** (2019).

**This column aims to highlight the personal perspective of a student. Therefore, the views and ideas expressed in this column are the own personal views of the columnist and do not necessarily reflect the view of RAMS. If you have any questions of comments regarding this column, contact the editorial board of RAMS.*

MYTH OR SCIENCE? MISSING: GIRLS WITH ATTENTION-DEFICIT/HYPERACTIVITY DISORDER

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I volunteer at two organisations where I teach children between the ages of six and eleven years old weekly. Somehow, at both of these organisations, there are many children (and volunteers) with neurodevelopmental disorders, including attention-deficit/hyperactivity disorder (ADHD). One of the intriguing aspects of this disorder is the difference in prevalence between boys and girls; ADHD is more prevalent in boys than in girls. Where does this difference in diagnoses between boys and girls come from? Are the girls who are bouncing around missed ADHD cases? Are dreamy girls who cannot remember what you just said displaying different characteristics of the same disorder? Or are there just more boys affected by ADHD than girls?

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder, meaning that the disorder has its origins in the development of the brain. Typical symptoms of ADHD include inattention, hyperactivity, and impulsivity [1]. In order to be diagnosed with ADHD, symptoms should have their onset before the age of 12 and should cause significant impairment in at least two areas of daily life, e.g. at school and at home [1]. About five per cent of children are diagnosed with ADHD, as opposed to two to three per cent of adults [2,3].

If I ask you to imagine someone with ADHD, the chances are that the first person that comes to your mind is a ten-year-old boy. This might be a stereotypical image; however, the representation is based upon truth, as ADHD is more often diagnosed in boys than in girls [4]. A European study found that the sex ratio (boys:girls) of ADHD diagnoses ranges from 3:1 to 16:1 in Europe [4]. Where do these differences come from? Are boys more susceptible to ADHD than girls? Are we missing girls in our diagnoses, or do girls have a different presentation of ADHD than boys? In this article, we will try to answer whether ADHD is genuinely more prevalent in boys than in girls or if there is another reason for this underrepresentation of girls.

History and symptoms of ADHD

Let us take a look at the history of ADHD. One of the first described ADHD cases is "Fidgety Philipp". He appeared in a children's book in 1864, written by Dr. Heinrich Hoffmann, a German psychiatrist [5]. This male fictional character displays all ADHD symptoms, predominantly hyperactivity [5]. Dr. Hoffmann also described two other characters, Johnny-Head-In-The-Air, who is very inattentive, and Flying Robert, who is impulsive [5]. Together, Johnny, Philipp, and Robert display all the ADHD symptoms. Other researchers describing ADHD at the beginning of the previous century found that more boys than girls had the symptoms they were focusing on, such as hyperactivity and impulsivity [6]. Currently, ADHD is seen as one disorder with three different presentations: the mainly inattentive presentation, the mainly hyperactive/impulsive presentation, and the combined presentation [1].

Underdiagnosis: sex-specific presentations

ADHD can be more difficult to diagnose in girls due to differences in the presentation of the disorder, and this underdiagnosis might be the cause of underrepresentation [7,8]. When we look at Johnny, Philipp, and Robert, it is evident that these boys present with very different symptoms and behaviours, although these boys have the same disorder. In a community sample, girls presented more often with internalising behaviour, such as inattentive symptoms and comorbid disorders, in comparison to boys [9]. Boys often display more externalising behaviour, such as hyperactivity and impulsivity [8,9]. Externalising behaviour is more easily picked up by parents and teachers than internalising behaviour, such as inattentiveness [7]. This leads to a phenomenon called referral bias, where boys are more easily referred to physicians than girls because the problems children with ADHD experience are less visible in girls than in boys [10]. One study by Mowlem *et al.* investigated which factors influence ADHD diagnosis in boys and girls [8]. The researchers interviewed a group of children with more severe ADHD symptoms than the general ADHD population and found that emotional problems most often determined if a girl with more severe ADHD symptoms was diagnosed with ADHD or not, an effect that was weaker in boys [8]. Moreover, parents rated impairments, such as conduct and peer problems, for diagnosed boys higher than for undiagnosed boys with severe symptoms of ADHD, but the same pattern did not show for girls [8]. Additionally, parents overestimated hyperactive/impulsive symptoms for boys in comparison with clinical interview data but underestimated these symptoms for girls [8]. This study shows how important the perception of parents and teachers is in order to make sure girls receive an appropriate ADHD diagnosis and get the help they need.

Perception of girls with ADHD

It is crucial to know how disorders, including ADHD, are perceived in society, as the perception of a disorder by society influences the acceptance of the disorder [11]. An interview with a representative population consisting of randomly selected adults, parents of children with ADHD, teachers, and adolescents, found that the majority of the included adults and teachers believe that ADHD is more prevalent in boys than in girls [12]. Moreover, the adults and teachers think that girls more often present with the inattentive presentation

instead of the hyperactive or combined presentation [12]. The most important result of this interview is the finding that 85% of the teachers and half of the general population and parents think that ADHD is underdiagnosed in girls [12]. Thus, underdiagnosis of girls is not only visible in research but also recognised by society.

The female protective effect

However, can underdiagnosis truly explain the underrepresentation of girls with ADHD? Recently, a new hypothesis, called the female protective effect, was formed to explain the differences in ADHD prevalence between boys and girls [15]. This theory assumes that there is a certain aspect of being female that protects you from developing ADHD. In other words, as a girl, you need more risk factors or a higher etiological load before you display symptoms of ADHD than boys do [15].

The female protective effect hypothesis is not completely new. Much more research on this theory has been done for autism spectrum disorders (ASD). In ASD, we see, just like in ADHD, more diagnoses for boys than for girls [13]. For ASD without an intellectual disability, the ratio (boys: girls) is 6-16:1, and with an intellectual disability, it is 1-2:1 [13]. Moreover, studies found that females with ASD often have more genetic mutations than males with ASD [13]. Furthermore, siblings of affected girls have higher ASD symptom scores and a higher risk of having ASD themselves than siblings of affected boys [14]. Additionally, the mutations that girls carry often have a higher impact considering that these are more closely related to the functionality of the gene network, meaning that the mutations have a higher impact on the biological pathways associated with ASD [13].

Following this line of thought, researchers wanted to investigate if they could find evidence for a female protective effect in ADHD. The first study to investigate this effect used a twin sample [15]. As ADHD symptoms vary with age, using twins was advantageous since they have the same age at the time of measurement. If being female is a protective factor, you would need to have more risk factors to develop a symptomatic disorder. If you are a twin of a female (sharing 50% of the genetic load), you are, therefore, likely to also carry more risk factors if you have a female twin with full symptomatic ADHD. A total of 10,759 dizygotic twin pairs from two cohorts were included and filled out a questionnaire about the presence and severity of ADHD symptoms. One twin from each pair was randomly selected as the index-twin, the other twin was the co-twin. The highest-scoring 5% and 10% on the questionnaire from the index-twins were selected. The researchers found that the co-twins of girls in the highest-scoring 5% and 10% cut-off groups had higher ADHD symptom scores than co-twins of boys in the corresponding highest-scoring groups [15]. This outcome was independent of the gender of the co-twin [15]. The effect is visible for total ADHD symptom scores, inattentiveness, and hyperactivity/impulsivity. Moreover, co-twins of affected girls were more likely to be affected themselves [15].

In conclusion, if you have a female co-twin diagnosed with ADHD or high ADHD symptom questionnaire scores, you are more likely to have ADHD or high ADHD symptom questionnaire scores yourself than when you have a male co-twin with ADHD or high ADHD scores. This study shows evidence that there might be a female protective effect. However, with one twin study, we have not nearly enough data to completely accept this hypothesis. Is this effect also visible for healthy siblings? Can we find a neural or even genetic basis to base our hypothesis on?

Finding further evidence

A recent study investigated the risk of developing a neurodevelopmental disorder in siblings of boys and girls with ADHD [16]. The study found that siblings of female subjects with ADHD had a higher risk of developing any psychiatric or neurodevelopmental disorder in comparison to siblings of male probands with ADHD. ADHD was the neurodevelopmental disorder that was most often diagnosed in siblings of the probands. Again, higher percentages of girls with ADHD were found to have a sibling with ADHD than boys with ADHD [16]. These results are interesting because we see evidence for the female protective effect both in a twin sample as in siblings. However, these findings still need to be supported by biological findings, such as molecular genetics or brain imaging, to fully explain the female protective effect.

If we look at the relationship between genetics and the risk of developing ADHD, the results differ between studies [17,18]. There is some evidence that siblings of girls with ADHD have a higher polygenic risk score than siblings of boys with ADHD. This finding indicates that siblings of girls have more low risk and more high-frequency genetic variants associated with ADHD than siblings of boys. However, in the diagnosed siblings, no difference in polygenic risk score was found for boys and girls. Overall, this means that girls have a higher familial genetic burden for ADHD than boys but do not necessarily have more genetic variants than boys [17]. Martin *et al.* suggest that ADHD in females could be more often associated with larger, more rare genetic mutations, which are associated with a worse clinical phenotype [17].

Considering that ADHD is a brain disorder, it would also be interesting to see if there are differences in brain structure and connectivity between siblings of boys and girls with ADHD. If the female protective effect on the risk of developing ADHD exists, we would expect that the brains of siblings from girls with ADHD are more similar to the brains of ADHD cases than the brains of siblings from boys with ADHD. Unfortunately, no research investigating this has been performed yet; nevertheless, it is a hot topic. In conclusion, there is evidence for a female protective effect on ADHD, but there is too little evidence to completely accept the hypothesis. This female protective effect would not be able to explain all of the differences in ADHD prevalence between boys and girls, but it can bring us one or a few steps closer.

Conclusion

Two theories might explain why ADHD is more often diagnosed in boys than in girls. The first theory concerns an underdiagnosis of ADHD in girls due to a different presentation of the disorder. This theory implies that the actual difference in the prevalence between boys and girls is much smaller than we now see or even non-existent. The second theory encompasses the female protective effect on the risk of developing ADHD. This theory implies that there truly is a difference in the prevalence of ADHD between boys and girls and that aspects of being female are protective for developing ADHD.

Which theory is true? Is there truly a difference in ADHD prevalence between boys and girls? We should keep in mind that the theories are not mutually exclusive. We are missing many girls in the ADHD diagnoses, and we should do better to give them the help they need. However, from the second theory, it seems plausible that females are protected, even though it is not yet clear how; much more research needs to be done to strengthen this hypothesis. When combining the two theories, there probably is an actual difference in ADHD prevalence between boys and girls due to the female protective effect, but this gap is not as large as it currently seems because of missed

diagnoses in females. Future research should, on the one hand, look into how we can recognise and diagnose ADHD better in girls and, on the other hand, look into the mechanisms behind the female protective effect.

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CORRECT ANSWERS TO THE EXAM QUESTIONS

Answer question 1:
A. Cytokines released by macrophages

During the start of an infection, macrophages release the cytokines IL-1, IL-6, and TNF-α, which stimulate the liver to produce acute-phase proteins. One of these proteins is C-reactive protein (CRP). CRP can bind to bacteria, yeasts, fungi, and several parasites, functioning as an opsonin and starting the classical route of activation of the complement system. Its serum concentration peaks within hours after the start of an infection. Therefore, the serum concentration of CRP is a relatively reliable biomarker for infections.

For further reading:
Parham, P. Chapter 3: Innate Immunity: the Induced Response to Infection in The immune system, 4th edition. (Garland Science, New York, 2015).

During the exam, 85% of the participants answered this question correctly.

The exam questions can be found back on page 20 in this journal.

Answer question 2:
A. APO lipoprotein allele epsilon 4 is necessary for fat metabolism at a younger age, whereas it increases the rate of developing Alzheimer's disease at an older age

Antagonistic pleiotropy is one of the proposed theories of ageing, stating that certain genes that promote our fitness during reproductive age can cause health problems at a later age, contributing to ageing. An example of this phenomenon is APO lipoprotein allele epsilon 4 that contributes to our health (fat metabolism) during our reproductive years but increases the risk of developing Alzheimer's disease later in life.

For further reading:
Stater, S., Saffitz, J., Rubin, E. Chapter 10: Aging in Rubin's Pathology: Mechanisms of Human Disease, 8th edition. (Wolters Kluwer, Philadelphia, 2020)

During the exam, 87% of the participants answered this question correctly.

ZEBRAS OF MEDICINE:
WHEN THROMBOCYTES ARE SCARCE

THROMBOCYTOPENIA IN LIVER CIRRHOSIS VS IMMUNE THROMBOCYTOPENIC PURPURA

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Abstract

Background: Thrombocytopenia describes abnormally low blood platelet counts in individuals. It is a common complication of numerous disorders and can be multifactorial in its cause. In liver cirrhosis, the origins of thrombocytopenia lie in a decreased liver function. Contrastingly, the low thrombocyte levels in immune thrombocytopenic purpura (ITP) are caused by autoantibodies targeting the blood platelets. A precise diagnosis of the patient is pivotal in order to select appropriate treatment.

Objective: This review aims to present the overlap and differences in symptoms between liver cirrhosis and ITP, raise awareness about the ambiguity between the two, and stress the importance of a proper diagnostic protocol.

Discussion: Thrombocytopenia is a key symptom in ITP, and up to 64% of cirrhotic liver patients present with abnormally low platelet counts. Furthermore, other cytopenias of leucocytes or erythrocytes tend to be absent in both conditions. In cirrhosis, the liver function is decreased, which becomes apparent following non-invasive diagnostic measures, such as imagining studies and biomarkers for liver function in the peripheral blood or following invasive measures like a liver biopsy. Regarding ITP, there are seldomly symptoms besides the abnormally low thrombocyte counts. The diagnosis is primarily a diagnosis of exclusion, making an unambiguous diagnosis more difficult. This diagnostic challenge stresses the importance of an in-depth investigation of the patient's health status. ITP can be distinguished in primary and secondary ITP, depending on whether the condition forms in response to a previous process or not (e.g. viral infection-induced or drug-induced). A clear distinction between the two conditions, ITP and liver cirrhosis, can substantially benefit the diagnostic process and improve the treatability of the condition.

Conclusion: In regard to liver cirrhosis and ITP, there is substantial overlap concerning symptoms. Furthermore, definite diagnostic measures for ITP are still lacking, making the process of diagnosing even more complicated. Nevertheless, a distinction between the two conditions is possible based on the patient's liver function and should be made in order to choose the appropriate form of treatment.

KEYWORDS: thrombocytopenia; autoimmunity; thrombopoietin; idiopathic thrombocytopenic purpura; platelets

Thrombocytopenia refers to a drastic abnormal lowering of blood platelet (i.e. thrombocyte) counts in the circulation [1]. The causes for this loss of thrombocytes are numerous and can be rooted in the production of thrombocytes, as well as their storage, functionality, and clearance [1]. This article aims to compare and delineate two causes for thrombocytopenia; liver cirrhosis being more common and immune thrombocytopenic purpura (ITP) being rarer.

Chronic liver diseases describe processes of continuous liver tissue destruction resulting in the replacement of healthy liver tissue by fibrous tissue, eventually leading to liver cirrhosis [2]. Globally, the number of liver cirrhosis cases increased by 74.53% between 1990 and 2017, with stark differences between countries [3]. Moreover, liver cirrhosis is ranked as the eleventh most common cause of death worldwide, causing approximately one million deaths in 2010 [2]. Common causes of this condition include hepatitis B and C, as well as alcohol abuse and non-alcoholic fatty liver disease [4]. Liver cirrhosis, more often than not, develops from an initial compensated form in which the liver can still carry out its necessary functions sufficiently, to a decompensated phase, where this is not the case anymore [4]. The classification of a patient into either the compensated or decompensated form is closely associated with his life expectancy, considering that median survival for a patient with compensated cirrhosis is considered to be over 12 years, whereas the median survival of patients with decompensated cirrhosis is merely approximately two years [5]. While the progression from compensated to uncompensated cirrhosis was traditionally thought to be an irreversible process, recompensation and even reversion of cirrhosis altogether have been described [4, 6].

Liver cirrhosis is strongly correlated with thrombocytopenia, as abnormally low platelet counts are found in as much as 64% of individuals diagnosed with liver cirrhosis, compared to 6% among non-cirrhotic patients with chronic liver disease [7]. The liver is crucial for the secretion of the hormone thrombopoietin, and, hence, the hormone levels are directly proportional to liver function [7, 8]. Thrombopoietin, in turn, induces differentiation into megakaryocytes (the progenitors of thrombocytes), while it also decreases platelet destruction through binding to matured thrombocytes [9, 10]. Thus, low levels of the hormone substantially impact thrombocyte levels, and loss of liver function indirectly reduces thrombocyte levels [10]. Furthermore, liver cirrhosis also directly augments platelet

destruction by a number of mechanisms, including heightened rates of platelet aggregation, immunologic destruction, and increased fibrinolysis [10]. Lastly, an increase in the blood pressure of the portal venous system (i.e. portal hypertension) induces thrombocytopenia via enlargement of the spleen (called splenomegaly) and, thereafter, an increased sequestering of thrombocytes in the spleen [11]. Thrombocytopenia is correlated with the long-term outcome of patients, which stresses the importance of proper treatment of thrombocytopenia for the cirrhotic patient [8, 12].

Different pathophysiological mechanisms are underlying ITP, which was previously also known as idiopathic thrombocytopenic purpura [10]. It has become evident that the pathophysiological processes are driven by autoreactive IgG antibodies targeting various platelet membrane proteins, like the glycoprotein VI [13, 14]. The binding of the autoantibody decreases the thrombocyte's half-life and interferes with thrombocyte production [14]. Nevertheless, the exact pathogenesis remains elusive and could also involve cytotoxic T cells attacking megakaryocytes in the bone marrow [14]. The autoantibodies can be drug- or illness-induced (thus, the associated ITP would be considered secondary ITP) or arise independently (then the ITP is categorised as primary ITP) [15].

Primary ITP can be further classified according to the duration of disease symptoms [15]. In the first three months following diagnosis, the disease is called newly diagnosed ITP. If the disease extends above this period for up to a year from the initial diagnosis, it is classified as persistent ITP. ITP persisting for more than 12 months from initial diagnosis is viewed as chronic ITP [15]. The epidemiology of ITP is thought to vary between age cohorts [16]. In 2009, the incidence of acute ITP (referring to incidences of ITP with durations of maximally 12 months) in children was investigated using previously published reports and estimated to be between 1.9 and 6.4 cases per 100,000 children [17]. In the same study, the incidence of ITP among adults was assessed and estimated to be between 1.6 and 3.9 cases per 100,000 individuals [17]. However, the underlying data are mostly derived from European countries, and, therefore, these results could be subject to geographical bias [17]. Most children only develop a newly diagnosed ITP and spontaneously recover again after three months, while adults tend to develop a chronic form of ITP [18]. Interestingly, sex disproportions among the patients vary too, according to the age of the investigated cohort [16].

While liver cirrhosis and ITP are very different in their pathogenesis, their overlap in causing thrombocytopenia and splenomegaly could cause confusion while diagnosing a patient presenting with low thrombocyte counts. However, up to now, no direct comparison between these two disorders has been drawn. This review aims to compare the clinical presentation, diagnosis, and treatment between both conditions in order to outline the common aspects, as well as the differences between the disorders. Furthermore, awareness should be raised that while ITP is much rarer than liver cirrhosis, it is still an important cause of thrombocytopenia and must, therefore, not be forgotten.

Clinical presentation

Patients suffering from liver cirrhosis often show little to no symptoms early on but will develop a number of symptoms at later disease stages. Early symptoms can include nausea, poor appetite, and mild abdominal discomfort, while later symptoms are more severe, such as jaundice and the collection of body fluids in the abdomen (i.e. ascites) and lower part of the legs and feet (i.e. oedema) [19]. Among cirrhotic patients, both hypersplenism (an overactive spleen) and splenomegaly are common complications [7]. These conditions, in combination with a progressively worse liver function, can lead to thrombocytopenia in the patient, and thus more frequent bleeding events [7, 19]. Histologically, cirrhosis presents as advanced liver fibrosis, distorting the surrounding liver vasculature [20]. Fibrosis describes the process of replacing damaged functional tissue with fibrous scar tissue, ultimately leading to loss of function of the organ if the fibrosis becomes excessive [20]. In the liver, occurring fibrosis increases the distance between hepatocytes and the nearest blood vessel, effectively cutting off hepatocytic islands from the blood supply [20].

Patients suffering from ITP, on the other hand, are generally well-appearing and present with only a few symptoms throughout the disease [16]. The strongest indicator is the thrombocytopenia itself, with a thrombocyte count of below 100,000/µl blood [16]. The counts of white blood cells, however, are in the normal range, as is the amount of haemoglobin, making the thrombocytopenia isolated [16]. Furthermore, patients show a generalised purpuric rash and may present with splenomegaly [16, 21]. However, the clinically most relevant symptom is bleeding of varying degrees, as it is a central cause of both morbidity and mortality [22, 23]. Strikingly, severe bleeding in the form of intracerebral haemorrhage occurs more in adults than children (1.4% vs 0.4%), while other forms of severe bleeding are more common in children than adults (20.2% vs 9.6%) [22]. Severe bleeding was associated with platelet counts below 20,000 platelets/µl blood, previous minor bleeding, and old age [22, 24].

Diagnosis

The diagnosis of liver cirrhosis is a multi-step process (Figure 1). Up until recently, liver biopsies were universally considered the gold standard of diagnosis of liver cirrhosis [4, 11, 25]. This procedure, however, is highly invasive and painful, shows an error rate of as much as 20%, and is not well suited for serial applications to determine disease progression [26-28]. Thus, other, less invasive diagnostic methods are currently under development [11]. Liver function (inversely correlated with liver cirrhosis) can also be determined non-invasively using biomarkers, such as serum albumin, cholesterol, cholinesterase, and coagulation factors [29].

Other approaches to the non-invasive diagnosis of liver cirrhosis are imaging methods, such as sonography, CT scans, and MRI scans [4]. In the past decade, sonography gained further importance due to the development of new applications like liver stiffness measurement and transient elastography (FibroScan) [4, 28]. Briefly, vibrations with a frequency of 50 Hz and mild amplitudes provoke a wave of shear stress that travels through the tissue. The velocity of the wave is measured and translates directly to the elasticity of the tissue [28]. Recently, it has been argued that transient elastography has the potential to eventually replace tissue biopsies as gold standards of diagnosis due to its non-invasive nature, low costs, and fast procedure, allowing for serial applications [25]. Furthermore, the procedure shows high accuracy in detecting advanced fibrosis of the liver, defined as stage F3 and above in a fibrosis classification system of F0 (no fibrosis) to F4 (cirrhosis) [30]. Advanced fibrosis is associated with an increased risk of worse clinical outcomes [31]. It needs to be noted, however, that this form of detection is negatively impacted by obesity, and the patient groups of the performed studies all showed a mean body mass index of maximally 30; thus, this diagnostic measure could potentially not be available for all patients [30].

After determining the presence of cirrhosis, the stage of compensation has to be determined next in a thorough examination of the patient [4]. In case of the presence of any potentially life-threatening complications such as ascites, sepsis, hepatic encephalopathy (brain

damage due to a reduced liver function and a subsequent build-up of toxins in the circulation), or thrombocytopenia, the cirrhosis is considered to be decompensated, and respective steps to treat the condition are taken [4, 11, 32].

ITP, on the other hand, is diagnosed primarily by exclusion criteria, following the observation of a platelet count of under 100,000 platelets/µl blood [16]. This lowered threshold (compared to the threshold of 150,000 thrombocytes/µl blood) is due to the fact that some individuals display low thrombocyte counts (between 100,000 and 150,000 platelets/µl blood) despite appearing healthy otherwise [15]. Laboratory evaluation should be performed to decide whether the thrombocytopenia is isolated, including differential laboratory evaluation, reticulocyte count, peripheral blood smear, blood type, and direct antiglobulin test. [33].

A crucial step in the diagnosis of ITP is deciding between primary and secondary ITP [16]. To this end, the history of the patient with respect to recent viral infections and received vaccinations should be clarified [34-37]. In light of the current pandemic of the SARS-CoV-2, it is interesting to note that secondary ITP was described as a complication following infection with SARS-CoV-2 in at least 45 cases up until September 2020 [38]. During the process of diagnosis, other causes of secondary ITP should be investigated as well, including drugs associated with thrombocytopenia, as well as related symptoms like weight loss, fever, or previous bleeding episodes of the patient [16].

Treatment

There are numerous approaches to treating liver cirrhosis, depending on the stage and severity of the condition and the presence of complications, which is why this article focusses on treating thrombocytopenia in cirrhotic patients [4]. A definite cure to chronic liver diseases, such as liver cirrhosis, are liver transplantations. However, while the cure rate nowadays is up to 80% in patients five years after transplantation, the waiting list can be extensive, and the costs are substantial [11, 39-41]. Thus, other treatment options should be considered [39, 40]. Another approach is a transjugular intrahepatic portosystemic shunt, which functions as a connection between the portal and hepatic vein, thereby relieving portal hypertension [42]. The procedure convinces with its low invasiveness and effectiveness against portal hypertension. Nevertheless, several severe complications (e.g. cardiac decompensation) make this procedure not suitable for all patients [43].

Up until today, it remains highly debated whether an increase in thrombocyte levels actually benefits the patient or whether the consequences of the procedure outweigh the health advantages [40]. On the one hand, platelet levels can be increased indirectly by targeting the spleen. Splenectomy, referring to the removal of the spleen, either in open surgery or laparoscopically via a small opening of the abdomen and a camera, remains controversial due to the bleeding risk in thrombocytopenic patients during surgery and the substantial morbidity of patients [44, 45]. Another approach is the partial splenic arterial embolisation, which is performed on cirrhotic patients presenting with splenomegaly and thrombocytopenia. In this treatment, the blood supply to small stretches of the spleen is impeded, resulting in their necrosis and, thus, a decrease in spleen size [46, 47]. The risk of complications rises with an increase in embolised spleen mass and includes pneumonia, peritonitis, and portal vein thrombosis [48]. The technique achieves results comparable to laparoscopic splenectomy but is associated with fewer risks [48].

Platelet levels can also be increased directly, either by platelet transfusions or administration of thrombopoietin receptor agonists.

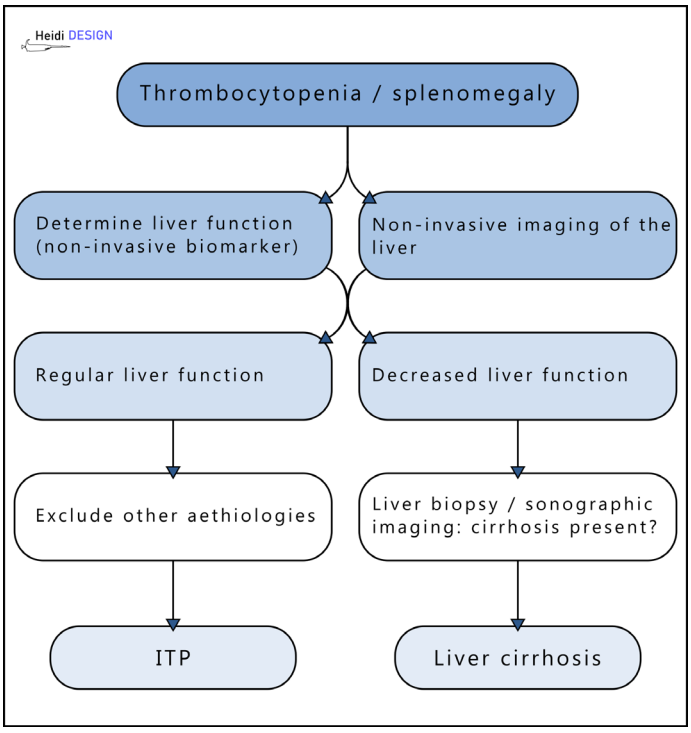

Figure 1: Flowchart of the differential diagnosis between liver cirrhosis and ITP.

Liver cirrhosis and ITP can both cause thrombocytopenia, as well as splenomegaly (an increase in spleen size). To distinguish these two conditions, the liver function must be determined using both non-invasive markers and imaging techniques, such as MRI. If the liver function is normal, ITP can be diagnosed by excluding all other possible conditions. If the liver function is decreased, the presence and extent of fibrosis can be determined using non-invasive sonographic imaging or invasive liver biopsies. ITP: immune thrombocytopenic purpura

Platelet transfusion improved liver regeneration in several *in vivo* studies using mice or rats, which is why its therapeutic potential for cirrhotic patients suffering from chronic liver disease-induced thrombocytopenia was investigated [40, 49, 50]. These patients were treated with platelet transfusions for 12 weeks, and both platelet counts, as well as liver function, were assessed in each patient [40]. The blood thrombocyte levels did not increase; nevertheless, the liver function increased markedly [51]. Values for serum albumin, serum cholinesterase, and serum hyaluronic acid showed a constant trend towards improvement, despite not being significant [51]. This increment could indicate a potential positive effect of platelet transfusions in liver cirrhosis [51]. However, the trial was non-controlled, non-randomised, and consisted of only a small patient cohort (six patients), and thus needs to be repeated in an improved study design including more patients to determine the clinical significance [40, 51]. Of note, platelet transfusions can cause serious side-effects, such as portal vein thrombosis and febrile reactions ranging from sole increases in body temperature to rigour and respiratory distress [40, 52, 53].

Hence, other methods have been explored to raise thrombocyte levels, including administration of thrombopoietin receptor agonists (e.g. eltrombopag, lusutrombopag, avatrombopag) [40]. Two agonists, lusutrombopag and avatrombopag, are currently approved by the US Food and Drug Administration for use in treating thrombocytopenia in adult patients with chronic liver diseases prior to them undergoing a surgical procedure [44]. A third drug, eltrombopag, has also undergone clinical trials but has not gained approval after

the observation was made that thrombotic events of the portal venous system were significantly increased in the treatment group over the control group (odds ratio of eltrombopag 3.04; 95% confidence interval 0.62-14.82) [52]. This discovery highlights the need for further research on eltrombopag and similar drugs and their association with thrombosis development in terms of determination of risk factors and dose optimisation. Until then, eltrombopag cannot be recommended as an alternative to platelet transfusion [52].

Treatments for ITP, on the other hand, can be classified into first-line and second-line treatments, the latter of which are administered if first-line treatment fails [16]. Generally, all patients should avoid antiplatelet therapies. Furthermore, patients with a platelet count of less than 20,000 platelets/µl blood should additionally stay clear off anti-coagulant therapies, and children with a platelet count of less than 30,000 platelets/µl blood especially should be discouraged from contact and collision sports and other activities associated with an increased risk of bleeding [16]. Generally, patients need to be monitored with respect to bleeding and their platelet counts [16]. About 50-70% of children recover spontaneously from ITP without the need for treatment [33].

First-line treatments primarily consist of the administration of glucocorticoids, namely prednisolone for children and prednisolone or dexamethasone for adults [33]. Children who cannot receive prednisolone can also be treated with either intravenous immunoglobulin or anti-D immunoglobulin [33]. If these first-line treatments fail or the ITP develops into a chronic form, second-line treatments can be administered [16]. For children, second-line treatments mainly consist of therapy with thrombopoietin receptor agonists, or if this fails, treatment with rituximab, an antibody that induces depletion of B cells [33, 54]. For adult patients, second-line treatments consist of thrombopoietin receptor agonists, rituximab, and finally, splenectomy [33].

Conclusions

Thrombocytopenia is a complex pathology and can have numerous origins. Thus, determining the exact cause of the thrombocytopenia is crucial for the most suitable and correct treatment. Thrombocytopenia as a consequence of liver cirrhosis has its cause in the liver and primarily in the reduction of thrombopoietin levels, while thrombocytopenia stemming from ITP is caused by autoantibodies targeting the blood platelets.

The two causes can be distinguished by determining the liver function and stiffness using biomarkers, non-invasive imaging techniques, or invasive liver biopsies, thereby determining whether the liver could be the origin of the low thrombocyte levels. This finding has imminent implications for the patient's treatment. The benefit of platelet transfusions to increase thrombocyte levels in cirrhotic patients is still debated among scientists, while increasing platelet levels in prolonged ITP is much more accepted. Thus, by utilising the outlined diagnostic measures, doctors can determine the origin of the thrombocytopenia and use this knowledge to provide the patient with the most befitting treatment.

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BORN THIS WAY OR CAN OUR BRAIN BE REWIRED?

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"Studying helps me to stay young." These are the words of Jan Warnink, who studied at the University of Leuven at the age of 80 years. He is not the only one taking this "it is never too late to start" spirit to a whole new level. In Italy, Giussepe Paterno received his bachelor diploma in history and philosophy at an impressive age of 97, and in Groningen, a lady of 88 years decided to study philosophy as well. Are they nuts for going to college as an elderly, or is there wisdom in doing a study after your retirement?

I was always told that if you want to achieve something, whether it was developing a skill or mindset, you should start at a young age. Once we grow old, we become more stubborn in our ways, and it is said that a large amount of effort is needed to get out of our comfort zone. However, this might not be so true after all. Although it was first believed that we are born with all the brain cells we will ever have, this idea has been proven wrong over the past years. It seems that our capacity to learn new things does not simply vanish once we grow old and that our brains are even able to grow new neurons over time. So, what does this mean? To what extent are we set in our ways when we are grey-haired? And, in the case of unlimited plasticity, are we able to influence the plasticity of our brain and become master learners?

The never-ending debate

Our brain is constantly reconnecting, creating new connections and strengthening existing ones. This so-called neuroplasticity improves communication inside the brain. At the core of neuroplasticity lies the process of growing new neurons and integrating them into neural circuits, referred to as neurogenesis. This process was always thought to only occur during the early life years [1]. However, more than a century ago, it was discovered that rats are capable of growing new neurons even during adulthood [2-4]. While this indicated that neurogenesis occurs in rodents, scientists had trouble figuring out the human situation. For over 50 years, studies on neurogenesis were bouncing back and forth on whether human adults neurogenesis existed or not. Until 2019, when Jason Snyder put a new, interesting idea forth [5]. He noted that labs investigating neurogenesis in mice used *young* mice, whereas human research was performed in *adults*. If we were to study humans and rodents relatively similar in age, we might find clarity about the existence of adult neurogenesis, or so he thought.

Although Snyder pointed out that the discrepancy in results between, as well as within, the rodent and human studies could be due to methodological differences, he was not convinced that adult neurogenesis in humans existed either. According to him, if adult neurogenesis does exist, then it exists at low rates and in very specific parts of the brain. Studies concentrating on the hippocampus found that rates of neurogenesis indeed varied throughout the lifespan in humans [6-8]. Human neurogenesis is found to be at its highest during the third month after we are born but persists throughout life at lower rates. However, why are neurogenesis rates lower at a later age? And, does adult neurogenesis serve a specific function?

Adult neurogenesis; function and significance

Nowadays, researchers are still not sure why and how newborn neurons are created. As the hippocampus is involved in learning and memory formation, some studies have highlighted the importance of newly generated neurons in learning, memory formation, and even in fear memories and spatial navigation [9]. One break-through article by Opendak and Gould dove into the literature and found evidence that silencing newly born neurons in adulthood results in memory impairment [10]. Opendak and Gould even suggested that newly generated neurons make connections with already existing ones [10]. It is thought that young neurons may help you adapt to a new environment or circumstances [10].

Adult life is full of changes; moving in and out of a new apartment, finding a new job, or changing your relationship status. It can be a struggle, and these challenges force us to be flexible and adapt to new circumstances. Of course, adapting to new circumstances requires us to learn from experiences and use this knowledge to our advantage. Newly generated neurons might help fine-tune the hippocampus, helping us adapt our responses to new environments [9]. For example, when shifting between jobs, you might be confronted with pesky co-workers, a condescending boss, or a demanding workload. Newly born neurons could play a key role in determining how you face those challenges.

Although the underlying mechanism remains unclear, the goal of adapting your responses to the new situations is more apparent, i.e. optimising survival strategies [10]. It is thought that newly born neurons help design a plan and prepare an individual on how to deal with stressful situations [11]. For an optimal survival plan it is important to know when to act and when to refrain. This skill requires one to distinguish between 'bad' and 'good' situations. This sounds easy, but it might be harder than you think. When we get older, it becomes harder to distinguish a person from a lookalike you met years ago. In order to do so, the hippocampus plays a crucial role in pattern separation: the process of distinguishing one memory from other, already stored memories [12, 13]. Deficits in pattern separation could lead to overgeneralisation of threats and the struggle to distinguish between safe and unsafe situations, which is often seen in patients with anxiety [14]. Mice with enhanced neurogenesis were better at performing pattern recognition tasks, and it has become clear that pattern separation depends on newly generated neurons resulting from neurogenesis [15-19]. The big question remains: can we influence adult neurogenesis and plasticity?

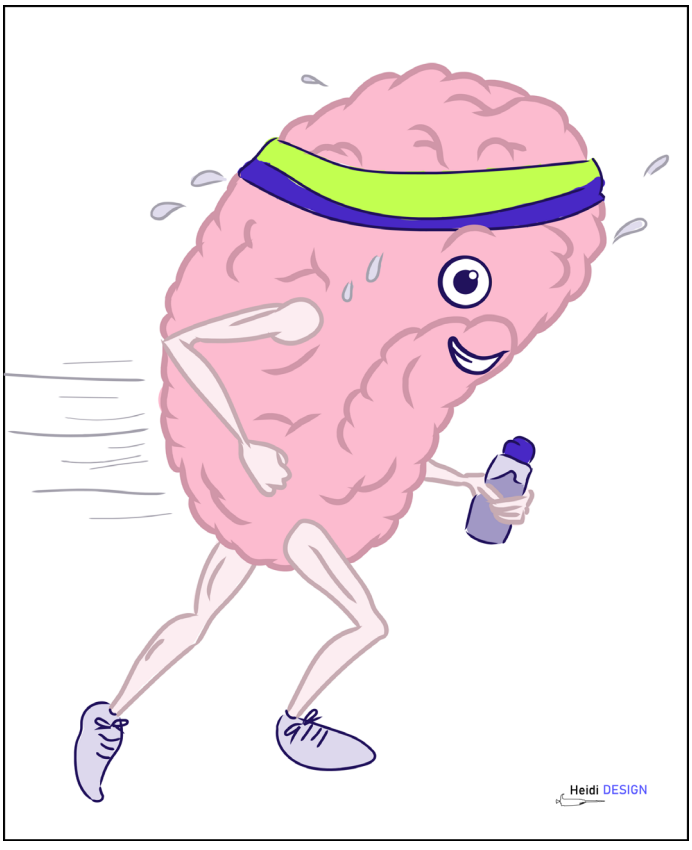
What influences hippocampal neurogenesis and plasticity?

In addition to Snyders observation that methodological differences might explain the discrepancy between and within human and mice research, two studies using methods similar to each other still found contradicting results. In 2005, post-mortem brains derived from different life stages (perinatal, postnatal, and adult samples) showed no newly born cells in hippocampal tissue [20]. However, these results are in contrast with a more recent study using a similar approach that observed both immature and newly adult-born neurons [6].

In other words, this last study found preserved neurogenesis. How could it be that these studies found contradicting results while using similar methods? It turns out there was a methodological difference all along. While the first study, reporting an absence of neurogenesis, obtained samples from individuals suffering from various diseases, the second study investigated samples of healthy individuals [6, 20]. What does this mean? Well, this means that diseases possibly disrupt neurogenesis and that a healthy lifestyle may be the key to prolonged neuroplasticity later in life.

Stress and antidepressants

Besides diseases, factors like ageing and stress do not benefit your brain condition either. The hippocampus is highly sensitive to stress, and this impact is even higher during periods of enhanced neuroplasticity, e.g. during infancy [21]. Hence, stress exposure in early life has been shown to have long-lasting effects on hippocampal neurogenesis and increases the susceptibility to developing disorders, such as depression, post-traumatic stress disorder, and Alzheimer's disease, later in life [22-25]. Additionally, patients with major depressive disorder show reductions in hippocampal volume, which may reflect reduced neurogenesis [26-28]. Moreover, hippocampal neurogenesis plays a key role in buffering stress responses in animals [29]. Hippocampal neurogenesis is currently investigated as a therapeutic target to improve the before-mentioned conditions, e.g. by antidepressants [30,31]. It is not exactly clear how, but antidepressants increase the expression of the *Brain-Derived Neurotrophic Factor* gene, which is needed for neurogenesis and plasticity [32-35]. However, this does not mean we should all run to the doctor to get a prescription for antidepressants. Lucky for us, there are other ways to increase your brain health.



Exercise

One way to promote neurogenesis and brain plasticity is by exercising. We already know that exercising is good for your physical health, but did you know it is good for your brain as well? A recent study in rats compared the effect of exercise on fear conditioning during adolescence and adulthood. Exercising rats had a higher expression of plasticity promoting genes, such as *Brain-Derived Neurotrophic Factor* [36]. Another study found similar results, reporting that rats that are exercising since adolescence showed an increase in amount, as well as complexity, of hippocampal doublecortin cells [37]. These immature neurons are used as a marker for neurogenesis. Moreover, exercise restores hippocampal plasticity and is associated with adaptive behaviour [32, 38, 39]. Exercise has both short- as well as long-term effects. For example, aged animals immediately improved their memory performance right after exercising, and sustained exercise boosted neurogenesis by promoting the connections between neurons and their integration into the communication network of the brain [32, 33, 40].

Environmental enrichment

Exercising is not the only way to improve your memory; putting yourself in a stimulating environment also influences the rate of neurogenesis [18]. This process is called environmental enrichment (EE), and it was first used by Donald Hebb, who raised rats in his home. He declared his pet rats were superior to laboratory-raised rats in terms of problem-solving abilities [41]. Nowadays, EE is not simulated by releasing rats in your home, but the animals are placed in big cages with small plastic toys, ladders, tunnels, running wheels, and sometimes a radio is placed near the cage. Scientists believe EE creates more opportunities to learn than a standard laboratory setting, and EE protects against age-related cognitive decline by stimulating neuroplasticity [42-46]. Moreover, a small period of only one week of EE is already enough to promote neurogenesis in mice [47]. Since the animals live in bigger cages, it is worth noting that an effect of exercising cannot be ruled out. Over the years, EE has remained largely a laboratory phenomenon, but it is currently investigated how these results translate to clinical settings. Maybe exposing yourself to a variety of experiences benefits your brain health as well.

Conclusion

After years of debate, the discussion on the existence of adult neurogenesis is finally settled. Human adult neurogenesis is real, and it is influenced by a number of factors, such as ageing, stress, exercising, and environmental enrichment. While ageing is inevitable, going for a walk, taking the time to relax, and challenging yourself to visit places you have never been before help to sustain the quality of your memory. Our brain is not a static organ and neither are we. So, whether you want to become a highly talented guitar player at a later age or want to make life-changing decisions, such as moving abroad or taking on a different career path: do not be afraid to do it! Your past experiences can help you handle difficult situations and help you thrive in whatever comes next, even if that is resuming a study after years of retirement. What is holding you back? Your brain certainly is not.

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RECENT HIGH-IMPACT PAPERS FROM RADBOUDUMC RESEARCHERS

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Summary

With over 3,000 publications each year, scientific research is a cornerstone of the Radboud university medical center [1]. In this section, recent high-impact papers with an impact factor higher than ten – published by researchers from the Radboudumc – will be discussed.

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A role for the innate immune system in cancer treatment

Immunotherapy is an anti-cancer treatment that makes use of the patient's immune system. The immune system consists of an innate and an adaptive part. As the innate immune system was believed not to have a memory, most immunotherapies target the adaptive immune system. However, recently, it has been shown that the innate immune system can be trained as well—trained immunity—, making it a major research topic within the research group of prof. dr. Mihai Netea (Department of Internal Medicine and Radboud Center of Infectious Diseases). In collaboration with other Radboudumc research departments, TU Eindhoven, and Mount Sinai Medical School New York, an article on trained immunity was published in *Cell* (impact factor = 38.6). A nanobiologic therapeutic, MTP10-HDL, was developed, and its effect on tumour growth was investigated in a melanoma mouse model (B16F10). MTP10-HDL significantly inhibited tumour growth by inducing trained immunity ($p = 0.0039$). In addition, via epigenetic mechanisms, MTP10-HDL stimulated bone marrow progenitors, resulting in more myeloid immune cells and increased cytokine production. These effects, in turn, resulted in a more pro-inflammatory tumour micro-environment (TME), favouring tumour destruction by the immune system. In addition, the effectiveness of checkpoint inhibitors, another type of immunotherapy, can be impaired by an anti-inflammatory TME. As MTP10-HDL treatment results in a more pro-inflammatory TME, adding MTP10-HDL improves the effectiveness of checkpoint inhibitor therapy. Finally, MTP10-HDL was not only found safe in mice but also in non-human primates. This offers possibilities for the application of MTP10-HDL as an anti-cancer treatment in humans. Before this clinical translation can be done, more extensive longitudinal studies using animal models are needed, which can also be used to investigate other types of cancers [2].

Does apathy predict cognitive deterioration or vice versa?

Apathy can be defined as a lack of motivation, initiative, and concern, and apathy is one of the main features of frontotemporal dementia (FTD), a well-known type of dementia. However, it has not been investigated before whether apathy is a predictor of cognitive deterioration in patients with FTD or the other way round. In collaboration with the University of Cambridge, prof. dr. Rogier Kievit (Department of Cognitive neuroscience, Donders Institute) answered this research question in *Alzheimer's and Dementia* (impact factor = 17.1). In this study, 304 carriers of mutations associated with FTD, as well as 296 of their relatives (non-carriers), were included. Study participants were pre-symptomatic and had a follow-up period of two years. The subjects underwent MRI scans and tests for apathy and cognitive function three times. Latent growth curve modelling was performed to assess relations between volumes of different brain regions, apathy, and cognitive decline. Apathy increased significantly during the period of follow-up in the carrier-group ($p = 0.004$) but not in the non-carrier-group ($p = 0.300$). In addition, apathy was found to be a predictor of cognitive deterioration in this group within two years ($p = 0.008$) but not the other way round ($p = 0.323$). Finally, low brain volumes of the frontal lobe and cingulate gyrus at the start of the study were associated with increasing apathy severity during follow-up in the carrier-group ($p = 0.038$ and $p = 0.037$, respectively). These results show that apathy could be identified as a marker of FTD-related cognitive decline. However, additional supporting evidence from studies with a longer follow-up period and larger sample size is required. Large sample sizes would also make it possible to investigate the previously reported findings in patient groups with different mutations separately. Together, those analyses could further increase insight into the role of apathy in the clinical course of FTD. Potentially, apathy could be an intervention target to decrease the risk of dementia [3].

Bone formation is less complicated than expected

The unique properties of bone tissue allow, among others, for organ protection, movement, and haematopoiesis. The traditional bone model assumes that bone consists of collagen fibrils and carbonated hydroxyapatite platelets (HAp crystals). According to this traditional model, the HAp crystals are organised parallel to the collagen fibrils in a structured deck-of-cards like manner. This organisation would presumably be guided by specific interactions with large biomolecules in the collagen. Sommerdijk *et al.* (Department of Biochemistry, Radboud Institute for Molecular Life Sciences) challenged this traditional model, and their results were recently published in *Nature Communications* (impact factor = 12.1). High-resolution electron microscopy confirmed that the HAp crystals were oriented parallel through the collagen. However, the orientation seemed to be random instead of in an organised deck-of-cards like manner. X-ray analysis revealed the structure of collagen, containing so-called gap regions. An *in vitro* model for bone growth showed that those gap regions provide HAp crystal organisation in the length direction of collagen. This type of crystal growth requires the least amount of energy, implying that crystal organisation is mainly determined by the laws of chemistry and not so much by specific molecular interactions. These results show that bone growth is less complicated than previously thought, as a highly complex matrix with specific biomolecules does not seem to be the main driver of crystal formation in bone anymore. This study opens doors for synthetic bone formation and regeneration, which, for example, could be valuable for patients with bone loss due to disease or fractures [4].

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RAMS

A Word from the Board of RAMS

Dear reader,

Thank you for reading the 18th edition of RAMS. We hope you enjoyed reading our latest edition, whether it was in physical form or online, and that you learned from this edition. This is the second release of RAMS' journal this academic year, and it once again contains a diverse collection of impressive articles written by our editors and submitted by our fellow students. I would like to thank everyone that contributed to this edition for their hard work.

This edition is the first RAMS edition of 2021, and, with the closure of 2020, we hope that a better future arrives. While we remain in uncertain times for now, the Netherlands started their vaccination program, which finally gives us an insight into when we might return to our pre-pandemic lives. Scientists have been working tirelessly since the start of the pandemic to develop vaccines that will prevent further spread of COVID-19. The crucial role of science in the development of vaccines confirms the societal importance of scientific research. Who knows, maybe you will be the scientist that saves millions of lives in the future.

For now, I hope you can find happiness in little things, such as a good movie, video calling your family and friends, or developing a new skill. If you enjoyed reading RAMS, you might be happy to know that we also have tons of online activities you can participate in, and we would love to bring you a little joy that way. I hope that emerging yourself in the wondrous world of scientific research gives you a little light in these difficult times and, remember, better days are coming!

Stay safe and take care.

On behalf of the board of RAMS,

Lotte van der Net
Chairwoman of RAMS 2020-2021

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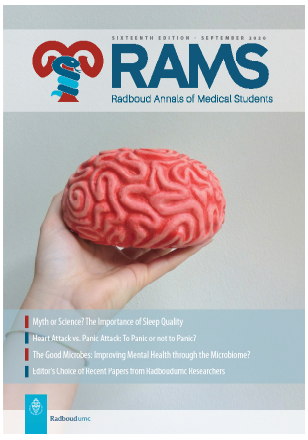

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