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FROM THE EDITORIAL BOARD

Dear reader,

As the days are getting shorter and the nights are getting longer, it is inevitable: it is becoming winter. This also means we are approaching Christmas already, my favourite holiday of all. Family gatherings, lots of good food, and hopefully some snow. With the very cold days ahead of us, we would almost forget that global warming is one of the biggest threats our dear Earth is facing right now. Global warming brings lots of difficulties and dangers with it, but have you ever thought about all the consequences of permafrost melting? Make sure to read the interesting piece in this edition, written by one of our editors, about this melting process, explaining the opportunities for new microorganisms to spread due to thawing permafrost. Could this cause the next pandemic?

All of our editors have managed to write various interesting articles for you once again. First, do not hesitate to read the piece about criminal minds, are they made or born? Next, make sure to read the article about long-acting antiretrovirals used as a treatment for HIV; could this be a breakthrough? Finally, do not skip the column about a medicine student running into an emergency situation on the street and having to apply his first aid protocol.

For now: snuggle into a warm hoodie and crawl onto the couch with this 21st edition. I wish you happy holidays and a very bright and educational 2022.

On behalf of the eighth board of RAMS,

Milou Serbée
Chair of the VIIIth Editorial Board

P.S. Are you studying medicine or biomedical science and working on your innovation project or research project, and do you think you have some exciting results that are worth sharing with the rest of the faculty? Do not hesitate to contact us. Or are you a molecular life science student or doing an MMD master and writing your thesis or another interesting piece of research? Then, again, please get in touch with us, as we can help you publish the best version of your article in RAMS!

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LONG-ACTING ANTIRETROVIRALS IN THE TREATMENT OF HIV-1

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Abstract

Although HIV has shifted from a death sentence to a manageable disease over the years, partly due to the development of highly efficacious antiretroviral therapies, its impact on the infected is lifelong. While there is currently a wide range of antiretroviral treatments available, the burden of HIV treatment remains high due to daily oral therapy. In the past decade, research into novel antiretrovirals for the treatment of HIV has slightly changed its course. Universities and pharmaceutical companies are now investigating and developing long-acting antiretroviral therapies. These long-acting antiretrovirals reduce the treatment burden by reducing the treatment frequency from once per day to once a week, once a month, or even once per six months. This search for novel compounds has resulted into two new treatment classes. With the first long-acting injectable antiretroviral treatment receiving market approval in 2020, the impact on the global HIV pandemic will manifest in the next couple of years. Long-acting antiretrovirals will decrease the treatment burden of the patient and likely increase treatment adherence, thus increasing the fraction of virologically suppressed HIV-patients. However, the cost of novel medication, an increased number of hospital visits for drug administration, and the mandatory oral lead-in period may result in a higher barrier of entry into the use of long-acting antiretrovirals by HIV-patients.

Introduction

Since its first reported cases in June 1981, the Human Immunodeficiency Virus (HIV) has grown to be a worldwide pandemic, which still claims about 680.000 lives per year to this day [1-3]. HIV is a lentivirus, which uses the CD4 protein on dendritic cells, macrophages, and CD4+ T-cells in combination with a chemokine receptor (most often CCR5 or CXCR5) to facilitate its entry into the cell (Figure 1). After its viral envelope fuses with the cell membrane, single stranded HIV RNA is turned into double stranded DNA by the enzyme reverse transcriptase (Figure 1) [4-6]. Subsequently, the newly formed double stranded DNA is integrated into transcriptionally active sites in the host's genome by the enzyme integrase [Figure 1] [4]. From that moment on, the virus will remain incorporated in the host cell's genome. The host cell will start producing new virus as soon as nuclear factors induce transcription in the locus where HIV was integrated. This new virus can then infect other cells in the host's body. If the infection is left untreated, the CD4+ population of cells will start to decrease, as HIV has various ways of accidentally killing its host cells [7]. If the CD4+ T-cell count falls below 200 cells/μL blood, the HIV infection progresses to a stage called acquired immunodeficiency syndrome (AIDS) [4]. Patients with AIDS have a much higher chance of developing opportunistic infections, as their immune system is much less functional [4, 6]. Historically, AIDS was seen as a death sentence, but with the development of increasingly efficacious antiretroviral (ARV) medication, HIV is manageable and has become a chronic disease.

The first ARV for HIV, zidovudine, a nucleoside-analogue inhibitor of the HIV reverse transcriptase enzyme, was brought to the market in 1987 [8, 9]. Since then, multiple ARVs in multiple drug classes have been developed to combat HIV. Combination ARV therapy uses multiple drugs with different mechanisms of action in the viral replication cycle. Currently, first-line treatment in treatment-naive patients often consists of two Nucleoside Reverse Transcriptase Inhibitors (NRTIs) and one drug, often an integrase strand transfer inhibitor (INSTI) such as dolutegravir, with a different mechanism of action [6, 8, 9]. HIV ARV treatments consist of pills, or solutions if the patient cannot swallow pills, which must be taken once or twice daily to achieve and maintain viral suppression [9, 10]. It is key to achieve and maintain viral suppression, as an uncontrolled HIV infection can develop into AIDS, and AIDS-related opportunistic infections may be deadly [10]. Additionally, the HIV reverse transcriptase enzyme is very error-prone and does not perform proof-reading [6]. These characteristics result in high replication and consequent mutation rates of HIV. If the patient has a non-suppressed HIV infection and sub-therapeutic exposures to ARVs, HIV may develop resistance against these ARVs and others in its drug class, which complicates treatment. However, due to the high pill burden, social stigma, and limited ARV availability in resource-limited settings, not all patients achieve viral suppression.

While traditional ARV regimens consist of daily oral pill intake, some pharmaceutical companies have spent the last years developing long-acting ARVs. These long-acting ARVs would, in most cases, eliminate the need for daily oral treatment and instead have periodical injections or other methods of drug delivery. Such a dosing regimen would promote treatment adherence and reduce the treatment load on the patient. There is currently one EMA-approved long-acting injectable treatment regimen, which consists of the integrase strand transfer inhibitor cabotegravir and non-nucleoside reverse transcriptase inhibitor rilpivirine [11].

Long-acting ARVs on the market or in development

Long-acting ARVs can belong to a multitude of different drug classes. Historically, the oral treatments have had a wide variety of mechanisms of action (Table 1, Figure 1).

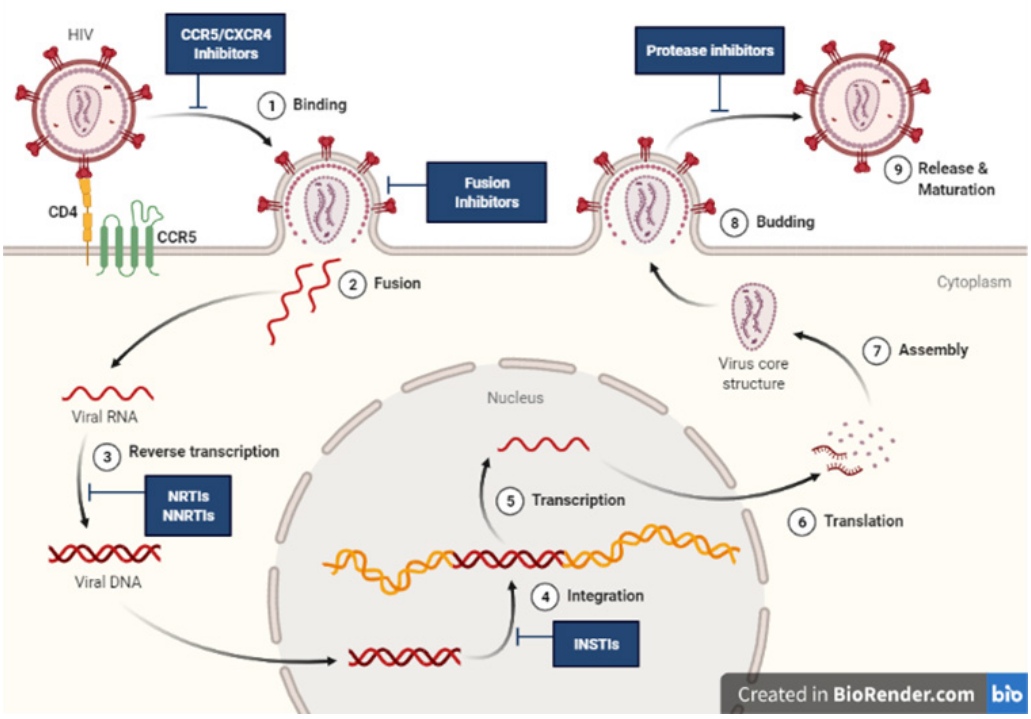

Figure 1: *Schematic overview of the HIV replication cycle. The replication cycle can be inhibited at several points. Successful HIV treatment makes use of a combination of at least two different modes of action (e.g., one drug inhibiting the reverse transcriptase/step 3 and another drug inhibiting the integrase/step 4). NRTI: nucleoside reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; INSTI: integrase strand transfer inhibitor. Reprinted from "HIV sites for Therapeutic Intervention", by BioRender.com (2021). Retrieved from https://app.biorender.com/biorender-templates.*

Table 1: *Overview of drug classes currently in clinical use for the treatment of HIV-1. NRTI: Nucleoside Reverse Transcriptase Inhibitor; RT: Reverse Transcriptase; HIV: Human Immunodeficiency Virus; NNRTI: Non-Nucleoside Reverse Transcriptase Inhibitor; PI: Protease Inhibitor; INSTI: Integrase Strand Transfer Inhibitor; dsDNA: double-stranded DNA; CD4: Cluster of Differentiation 4; ARV: Antiretrovirals*

Drug class	**Mechanism of action (Figure 1)**	**Examples (not all are shown)**
NRTI	The HIV enzyme RT is necessary to turn single stranded HIV RNA into double stranded HIV DNA. NRTIs are nucleoside-analogues. As the RT-reaction is taking place, the NRTIs are built into the new DNA. However, NRTIs lack the 3'-OH group, which is necessary to keep building the new DNA strand. Incorporation of an NRTI-molecule leads to termination of the RT-reaction, and an incomplete or non-functional piece of HIV DNA. [9, 12]	Zidovudine/azidothymidine (1987) Lamivudine (1995) Abacavir (1998) Tenofovir disoproxil fumarate (2001) Emtricitabine (2003) Tenofovir alafenamide (2015)
NNRTI	NNRTIs also act on the RT enzyme, but with a different mode of action. The RT enzyme is structurally changed after an NNRTI binds in the NNRTI pocket. Although this does not fully stop the enzyme's activity, the change results in such a reduction of function that the NNRTIs are a highly effective class. [9, 12]	Nevirapine (1996) Efavirenz (1998) Etravirine (2008) Rilpivirine (2011) Doravirine (2018)
PI	The protease enzyme is needed to cleave immature HIV into its mature variant. PIs block the active site of protease and thus inhibit the formation of mature HIV and its subsequent release from the infected cell. [9, 13]	Saquinavir (1995) Ritonavir (1996) Atazanavir (2003) Tipranavir (2005) Darunavir (2006)

Fusion Inhibitors	Fusion inhibitors disrupt the process of viral entry into the cell by binding to the gp41 subunit of the envelope glycoprotein. As a result, a six-helix bundle, which brings together the viral and cellular membranes, cannot be formed, and the virus cannot enter the cell. [9, 14, 15]	Enfuvirtide (2003)
CCR5 Antagonists	CCR5 antagonists bind to the human chemokine receptor CCR5. CCR5 is one of the chemokine co-receptors that HIV can use to gain entry into the cell. By blocking the receptor, HIV cannot make use of this. However, other variants of HIV could make use of other chemokine receptors, such as CXCR5. [9, 15, 16]	Maraviroc (2007)
INSTI	INSTIs block the workings of the integrase enzyme. As a result, the newly formed HIV dsDNA will not be incorporated in the host's genome. [9, 17]	Raltegravir (2007) Dolutegravir (2013) Bictegravir (2018) Cabotegravir (2021)
Attachment Inhibitors	Attachment inhibitors prevent HIV from binding to CD4 by directly binding to the viral envelope gp120. This appears to be the earliest step in which HIV can be combatted medicinally, apart from neutralising antibodies. [9, 15, 18]	Fostemsavir (2020)
Post-Attachment Inhibitors	Post-attachment inhibitors bind to CD4. Although binding of HIV to CD4 is not prevented, post-attachment inhibitors prevent the subsequent co-binding to chemokine receptors, preventing HIV entry into the cell. CD4 retains its immunological function when post-attachment inhibitors are used. [9, 15]	Ibalizumab (2018) Note: Ibalizumab (and most other drugs in this class) must be administered intravenously and cannot be taken orally.
Pharmacokinetic Enhancers	Pharmacokinetic enhancers generally alter liver enzyme activity, which would normally metabolise other ARVs. This way, the same dose of ARV can lead to a higher or longer exposure to the drug, resulting in higher efficacy but also risking toxicity as a result of overexposure. [9]	Cobicistat (2014)

Current oral HIV treatment has been efficacious for treatment-adherent patients. However, some patients are unable to swallow pills, struggle with treatment adherence, or have an altered absorption state, rendering the standard oral treatment ineffective for them. Logically, pharmaceutical companies have turned to ARVs that are already in use for the treatment of HIV to create long-acting treatment variants (Table 2). However, some drug classes have more suitable characteristics to be used as a long-acting treatment for HIV, such as a favourable resistance profile or a longer elimination half-life. Due to the large number of antibodies that are in development against HIV, these are not described in more detail in this article [19]. The search for suitable compounds for long-acting treatment has also yielded some novel drug classes, such as nucleoside reverse transcriptase translocation inhibitors and capsid inhibitors [20].

These long-acting ARVs offer various treatment options for both treatment-naive patients as well as patients that seem to have exhausted most other ARV options. While most long-acting ARVs have not set a definitive optimal dosing regimen, all long-acting therapy will relieve (some) pill burden off the patient. The dosing intervals are tested as short as one week (islatravir), one to two months (Cabenuva), to six months (lenacapavir). While this increased dosing interval eases the patient's treatment burden, the hospitals will have an increased workload, as patients must visit the hospital for injections and implants. However, a lot can change before regulatory approval, and, with more study time and possibly the use of pharmacokinetic enhancers, the dosing interval can perhaps be prolonged further for some of the long-acting ARVs.

Table 2: *Long-acting treatments currently in development. Neutralising antibodies clustered, as a multitude of companies are investigating several antibodies in different stages of clinical development. IV: Intravenous; INSTI: Integrase Strand Transfer Inhibitor; NNRTI: Non-Nucleoside Reverse Transcriptase Inhibitor; IM: Intramuscular; NRTI: Nucleoside Reverse Transcriptase Inhibitor; NRTTI: Nucleoside Reverse Transcriptase Translocation Inhibitor; PrEP: Pre-Exposure Prophylaxis; PI: Protease Inhibitor; SC: subcutaneous; TAF: Tenofovir alafenamide; IP: Intraperitoneal.*

Compound name(s)	Drug class	Formulation	Clinical development phase and dosing frequency
Albuvirtide	Fusion inhibitor	IV infusion	Phase III (TALENT; NCT02369965): Once per week
Atazanavir	PI	Injectable (SC, IM)	Preclinical
Cabenuva (Cabotegravir + Rilpivirine)	INSTI + NNRTI	Injectable (IM)	On market: Once per 1-2 months
Combinectin	Entry inhibitor	Injectable (SC)	Phase I (NCT03984812): Once per week
Elsulfavirine	NNRTI	Oral; Injectable (SC, IM)	Phase Ib (NCT03730311): Once per week; Preclinical
GS-CA1	Capsid inhibitor	Injectable (SC)	Preclinical
GSK'937	Maturation inhibitor	Injectable (SC, IM)	Preclinical
Islatravir (EFdA)	NRTTI	Oral; Subdermal implant (PrEP)	Phase III (Impower-024; NCT04652700): Once per month; Phase I
Lenacapavir	Capsid inhibitor	Injectable (SC), oral	Phase III (CAPELLA; NCT04150068): Once per 6 months
Raltegravir	INSTI	Injectable (SC)	Preclinical
Ritonavir	PI	Injectable (SC, IM)	Preclinical
TAF	NRTI	Subdermal implant	Phase I/II (CAPRISA 018): Once per 6 months
Various neutralising antibodies	HIV-1 envelope protein antibodies	Injectable (IV, IP)	Various stages of development

The long-acting ARVs are not only being tested as treatment. Some of the compounds may hold the potential to exhibit a protective function against an HIV infection as well [21, 22]. In such a case, the drug must be taken before participating in activities that put the individual at risk of an HIV infection, in a treatment method called pre-exposure prophylaxis (PrEP) [21, 22]. Such PrEP treatments are already available as daily oral pills – most commonly in the form of tenofovir disoproxil fumarate paired with emtricitabine, or both combined in a non-generic fixed dose tablet– but these exhibit a large pill burden on the individual. In order to remain safe, the treatment must be taken each day during high-risk behaviour periods, such as sexual activity in men who have sex with men. Reducing the burden of treatment adherence by replacing daily oral PrEP with any of the long(er)-acting ARVs can offer easier, more reliable protection to those frequently at risk of an HIV infection [21, 22].

Discussion

As the pathology of HIV is understood better over the years, a much more targeted approach can be taken to combat HIV. The global effort to combat the novel viral infection in the eighties resulted in the repurposing and regulatory approval of a drug within a couple of years after the discovery of HIV. What followed was twenty years in which therapies to treat HIV in new ways were presented on the market every year or two. Now that basic, effective treatment has been established, the next generation of anti-HIV medication can focus on other aspects of the disease, such as reducing possible side effects of the medication, reducing the pill burden, and effective prevention of HIV infection.

The upcoming long-acting ARVs offer current patients a reduction in their daily disease burden or new treatment options if other options have been exhausted.

However, there are limitations to the long-acting ARVs. Although the daily burden of the disease is decreased, the importance of treatment adherence is increased. Missing injections or pills will result in a subtherapeutic concentration of the ARV, allowing

for a viral rebound in the blood HIV RNA count. This may, in turn, lead to novel mutations in HIV and result in treatment resistance, rendering the long-acting ARV and possibly other (oral) drugs in the same drug class useless for this patient. In the same manner, stopping the treatment and switching to a different treatment regimen is not without risk of developing treatment resistance, as the currently approved regimen of cabotegravir and rilpivirine can be detected in blood up to a year after the last injection.

Next to this, there are some problems related to care organisation. If patients start using the long-acting injectable ARVs, these must be administered in the hospital or clinic which will result in additional work for the hospital or clinic. This would also require the hospital or clinic to train additional staff to administer these injectables. The injectables must remain cooled from production until the moment of administration in the patient. Together, these points limit the impact long-acting injectable ARVs could have in resource-limited settings.

Another limiting aspect comes in the form of the oral lead-in period for the injectables. This oral lead-in period is used to test for hypersensitivity and other adverse events related to treatment in the patient. As the long-acting ARV injectables will release their active substance for a long time, the patient could face long persisting negative effects of the medication if they are hypersensitive. The oral lead-in period raises the barrier of entry into long-acting ARV injectables, as not all patients may be willing to put in the work to closely monitor their health for up to a month or may be unable/unwilling to swallow new pills for a month. However, there is no consensus on the use of the oral lead-in period. Following an update to the summary of product characteristics, this lead-in period is now optional for the cabotegravir and rilpivirine combination injectable treatment. It is not yet clear how other treatments will tackle this problem.

As these long-acting treatments are new, there has not yet been ample time to build clinical knowledge regarding special patient populations, such as children or pregnant women, and interactions with other drugs. Safety data regarding pregnancy and children are more difficult to gather and will take a long time, as low concentrations of the long-acting treatments will remain present in the body for a long time, even after discontinuation of treatment. Next to this, the clinical application of long-acting treatments may be limited by the nature of HIV treatment. It is difficult to find partner drugs for long-acting treatments. These partner drugs must be of a different drug class and preferably be dosable in the same interval. Combine this with patient-specific resistance or side effects, and the development of new long-acting treatment regimens is now a lot more difficult.

The final limitation of long-acting ARVs comes at its price. Currently, only one option is on the market to be used as a full treatment regimen. Disregarding the price of the oral lead-in period of 28 days and the increased first injected dose, maintenance doses will cost $1,036, - (€895.22) per month and are likely not covered by insurance. For reference, a commonly prescribed oral combination pill of non-generic doravirine, lamivudine, and tenofovir disoproxil fumarate will cost €530.40 per month, which is covered by insurance in The Netherlands. While long-acting ARVs seem to be the way forward for HIV treatment and prevention, time will tell if these are the way forward for HIV patients.

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LOOKING INTO THE FUTURE

PATHOLOGY AND ARTIFICIAL INTELLIGENCE: A DREAM TEAM?

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Insight

The field of pathology encompasses the understanding and visualisation of illnesses on a cellular level, allowing for the specific characterisation of disease. For this reason, pathology is essential in clinical care. Nevertheless, despite its necessity, only a minority of the students choose this profession. The field of pathology will change in the future, mostly because of the exciting opportunities for the application of artificial intelligence in pathology. It is clear that this shift is approaching fast. However, when and to what extent remains undefined. Will artificial intelligence have received a prominent place in the doctor's office by 2050, or will the translation to the clinic prove to be more difficult?

Pathology is –arguably– the backbone of the diagnostic process, as the examination of histological slides often provides the highest level of certainty about a diagnosis. Especially in oncology, determining the type of malignancy, often including genetic testing of the tissue, is indispensable. Up until five years ago, the histological slides were predominantly examined through the microscope by pathologists. However, in the past five years, pathology has started to transition from microscopy to digital pathology, meaning that the histological slides are scanned and stored on the computer [1]. Pathologists can then examine the slides digitally and even share them online with colleagues. The online images of the histological slides are referred to as Whole Slide Images (WSI) [1]. The implementation of these WSI has opened the door to applying artificial intelligence (AI) in pathology to aid the examination of these slides [1]. The possible uses of AI are auspicious: however, it also raises questions about its future applications. How impactful will AI eventually be in the diagnostic process? Will we receive our diagnosis from an algorithm, a pathologist, or both?

The history of deep learning

Artificial intelligence refers to any intelligence or cognition that a machine displays, similar to the intelligence of animals, such as humans [2]. There are multiple forms of AI, one of which is deep learning [2, 3]. Deep learning itself has emerged in the past ten years and has been applied extensively in other disciplines, such as radiology, speech and image recognition, and pathology. An important enabler for deep learning in pathology is the widespread implementation of digital pathology [1]. One of the barriers to deep learning research, however, is the need for large annotated datasets of WSI to adequately train the algorithms [1, 2]. Nevertheless, in the past few years, more and more datasets have become publicly available, increasing the accuracy of the algorithms, thereby increasing the applications of AI. The computational pathology group of the Radboudumc has been at the forefront of sharing annotated datasets publically, *e.g.* through the CAMELYON challenge in 2016 and 2017, where the research group put a dataset of WSI containing annotated metastases of breast cancer in lymph nodes online [1, 4]. The contest was eventually won by Harvard University and MIT out of 32 participants [1]. During the CAMELYON challenge, the group of Harvard already designed algorithms that could outperform the pathologist used in the challenge. Since then, more and more algorithms have been designed that performed equally to experienced pathologists – and in some cases, even outperformed pathologists [1]. In other words, the AI algorithms enabled better image analysis in certain tasks compared to a trained pathologist [1, 4]. Should this mean that AI needs to take over the profession of being a pathologist to increase adequate patient management?

Computational pathology and deep learning

In order to discuss the future role of deep learning in pathology, we have to get technical. In short, computational pathology concerns the implementation of deep learning algorithms for analysing histopathological images [2]. Computational pathology uses algorithms to analyse slides. The algorithms are developed in order to achieve a specific output, *e.g.* detection of metastases in lymph nodes (Figure 1). The algorithms are trained using datasets of WSI, which are annotated by professionals (mostly pathologists), defining the desired output of the model [2]. If the algorithm is designed to detect metastases in lymph nodes, for example, the input will be WSI, in which the area with metastases will be annotated. In most cases, training will include cases with normal tissue as a control group.

The deep learning algorithm, which uses a neuronal network, operates directly on the WSI pixels, in order to analyse its input. The neuronal network consists of layers of connected neurons, in which, starting from the pixel values, activations are calculated for each consecutive layer on the basis of the values in the previous layer(s). (Figure 1). You can envision this as the connections in our brain – our neurons receive input (the pixels) and connect with thousands of other neurons. In the algorithm, it is not just the value of one pixel that is important but also the connections between the pixels [2]. The nucleus/cytoplasm ratio, for example, is of importance when detecting cancer and can be determined by differences in the intensity of the HE-stain between different parts of the cell; which includes multiple pixels. The algorithm analyses these connections between the pixels in the neuronal network and assigns to each connection a value depending upon the importance of the connection for the outcome of the algorithm. This is a step-wise process, where the algorithm

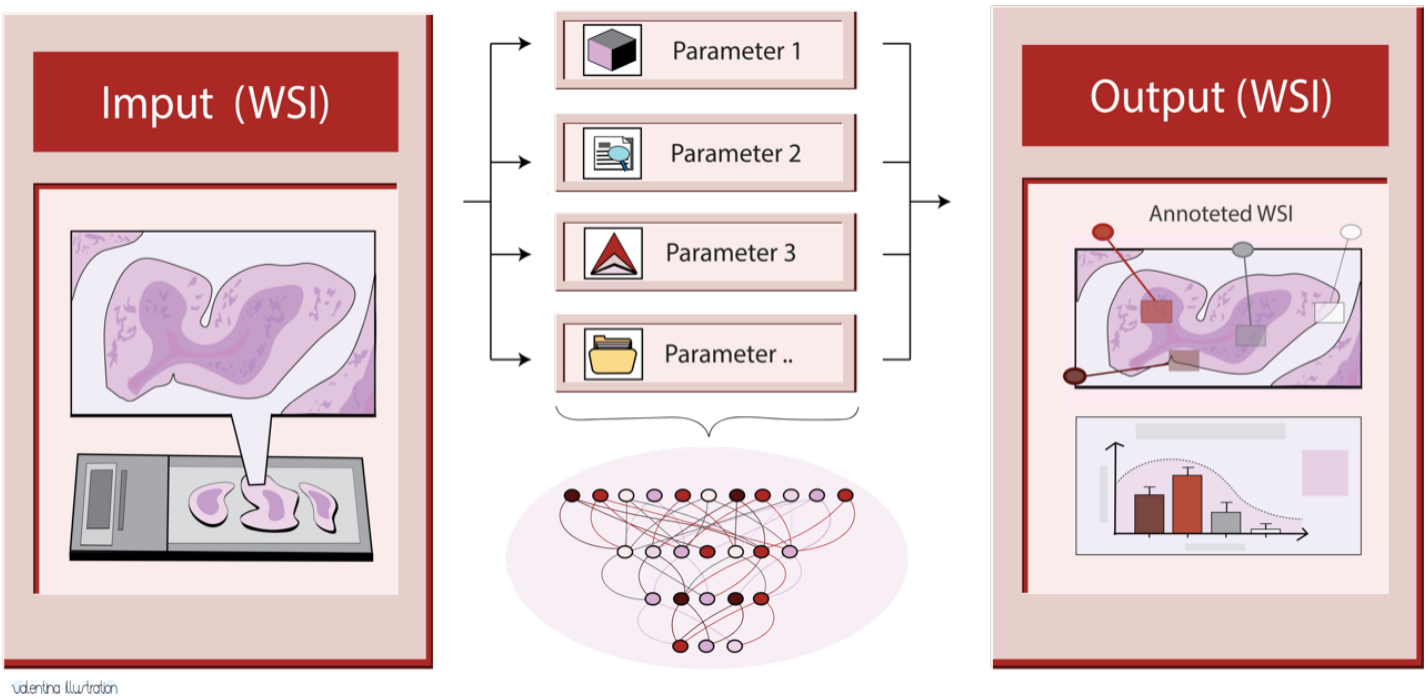

Figure 1: *The algorithm identifies areas of interest (depending upon the algorithm's purpose), through analysis of the connections between pixels. This analysis is achieved by applying a neuronal network to the image, where different layers of the neurons analyse the connections. This is a step-wise process where the value (importance) of previous connections between pixels and/or other connections in which the pixels are involved are also taken into account. Thus, the algorithm narrows down the areas of interest step-by-step, eventually leading to the desired output.*

narrows down the number of connections to those of importance [2]. Thus, the algorithm determines a set of parameters that are essential for establishing the output. Those parameters can then be applied to new WSI after proper training of the algorithm. The algorithm's performance is checked by running it on a second, independent data set and comparing its outcome with the performance of experienced pathologists.

The advantages of deep learning

One of the most significant advantages of applying deep learning is that AI is consistent in its analysis of the slides, minimising inter-observer differences. The algorithm functions the same regardless of which slides it analyses and should consistently provide the correct output. However, if a pathologist analyses the slides, you will always have a certain degree of disagreement and inter-observer differences [1, 2, 4]. Furthermore, the algorithms can analyse data faster than a pathologist [1-4]. Thus, its application comes in handy when there is ever-more tissue that needs to be analysed. For example, deep learning could screen large amounts of slides, which would be useful in nationwide screening programs. These programs, such as the screening programs for colon cancer, and cervical cancer, often generate many tissue samples that take quite some time to analyse and where the predominant part will not contain malignancies [1]. Conversely, the algorithm could mark any WSI where malignancy is suspected, which a pathologist can then analyse, saving a lot of time [1, 2].

Furthermore, deep learning can be used for quantitative tasks, *e.g.* determining the amount of tumour-infiltrating lymphocytes, mitosis hotspots, or programmed death-ligand 1 expression, therefore being able to also determine a wide range of biomarkers that are too work-intensive to be determined by the pathologist [4]. By obtaining more information about these biomarkers, we might even be able to better correlate these biomarkers to clinical outcomes and include more biomarkers in our prognosis or treatment choice. In addition, deep learning can pick up more subtle changes in an image, those which human eyes often overlook. An example of this is tumour budding, which refers to a single or a few cancerous cells at the edges of a tumour, indicating a more invasive character [1, 4]. Recently this has received increased attention, as it can influence prognosis.

The disadvantages of deep learning

This sound promising, right? So why has deep learning not been applied more extensively then? Well, one of the most significant issues with deep learning is generating enough annotated input to train the algorithms [1]. Considering that the slides must be annotated manually, it takes many man-hours to obtain enough annotations to train the algorithm. Multiple approaches to decrease the workload have been tried, such as using medical students [1]. However, in the end, a professional –often a trained pathologist– needs to check the annotations to ensure their quality, which results again in a time-limiting factor [1].

Another challenge is the lack of generalisability of the algorithms [1, 2, 4]. The algorithms often perform well, sometimes even better than pathologists, on slides of the same dataset used for training. These are often slides scanned by the same scanner and the same protocol in a similar patient population in one hospital. However, once the algorithm is applied to another dataset with variations in patient characteristics or the scanning process, the performance decreases. [1, 5]. Therefore, the quality of the algorithms depends on the type of scanner and the hospital where images were made. Thus, the algorithms are first validated internally, *i.e.* tested on the same dataset the training images were derived from, after which it is validated externally, *i.e.* tested on another dataset, different to the one used for training [1]. In order to prove its final accuracy, the algorithm should be tested in a clinical trial where it can be compared to pathologists in a real-world diagnostic setting [1]. However, most

of the clinical trials using AI were tested in different fields than pathology [1]. In addition, several AI programs have been approved by the Food and Drug Administration (FDA), but thus far, only one is applicable within pathology [6, 7]. One of the issues underlying the limited implementation of AI programs is the difficulty in validating the algorithms in accordance with the current guidelines – and the algorithm can not be tested in clinical trials before it has been validated internally and externally [1, 2, 8-10].

Looking into the future

So how will the future of pathology look like? What will be the work of the pathologist in 2030? Although computational pathology is often speculated to replace pathologists, this is most likely not a realistic scenario. Nevertheless, the algorithms could very well support pathologists in their tasks [1, 2, 4]. They will most likely be implemented by the year 2350, especially for the quantitative tasks or applications that comprise repetitive tasks (e.g. screening colon polyps for abnormalities). However, pathology encompasses more than merely classifying tissue. It also includes interpreting data in light of the clinical presentation and symptoms, establishing a prognosis, and clearly communicating results to other clinicians. Although algorithms might achieve this someday, I do not believe this being easily reached in 2050, especially when considering ethical and legal ramifications. Speaking of which, would the general public be open to receiving a diagnosis made by an algorithm? Moreover, who would be responsible if the algorithm made a mistake [11, 12]? In the Netherlands, the physicians registered in the Dutch registry of medical doctors are legally accountable for their mistakes. The physician can receive an official warning or even be removed from the Dutch registry after an error and, in that case, be forbidden from performing their clinical work. However, an algorithm is not part of the Dutch registry, so who is legally responsible if an algorithm would make diagnoses independently without final control by a pathologist [11, 12]? All in all, although algorithms will be beneficial, perhaps even indispensable in the future, they will -most likely- not fully replace pathologists.

Conclusion

Deep learning has been extensively researched in the past few years and has been shown to be effective in pathology. In some cases, algorithms even outperform pathologists for well-defined tasks. Therefore, algorithms could support pathologists and help with analysing quantitative data as well as large amounts of data, especially taking into account that algorithms are faster than pathologists. However, it is not all sunshine. The decreased performance of the algorithms on datasets from those used for training currently limit its clinical application. Furthermore, the generation of enough annotated data remains a barrier in the widespread implementation of deep learning. Even if algorithms will continue to be established that are equally as effective as pathologists, ethical and juridical consequences will arise. Nonetheless, the field of pathology will undoubtedly have changed by the year 2050; deep learning will probably have earned its place in the standard practice of pathology.

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THE LAMP AT THE END OF THE TUNNEL

LOOP-MEDIATED ISOTHERMAL AMPLIFICATION ASSAY (LAMP) AS A POTENTIAL ALTERNATIVE TO PCR-BASED DIAGNOSTICS

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Abstract

Diagnostic testing is key in mitigating the spread of infectious diseases. Although current nucleic acid amplification tests such as PCR-based tests are usually highly accurate, they utilise expensive equipment and complicated processes. These qualities make it largely inaccessible, especially to low- and middle-income countries. An emerging technology called loop-mediated isothermal amplification (LAMP) fosters the potential to be an accessible, cost-effective, yet comparably accurate alternative to existing gold standard methods. LAMP is a rapid single-tube technique that amplifies the target gene at a constant temperature, thus eliminating the need for the expensive thermocycler. Results can be determined by simply looking at a colour change in the sample. Furthermore, it only requires assay reagents and a heating source such as a water bath or a heat block, which are suitable for low-resource settings. This method has already been deployed in the field of primary care settings for COVID-19 and other diseases. Although the assay still leaves room for improvement, it has the potential to provide diagnostics more equitably and control outbreaks more efficiently.

Whether it is for COVID-19 or other infectious diseases, diagnostic testing is a ubiquitous public health tool. Diagnostics play a pivotal role in containing the spread of outbreaks and endemic diseases [1]. However, in resource-limited areas, the main challenge is accessibility to these diagnostic tests that consequently result in the delay of initial detection of community transmission. This diagnostic bottleneck has been linked to the accelerated spread of recent outbreaks such as SARS-CoV-2, Ebola, Zika, and yellow fever [1, 2]. The poor level of surveillance delays the time to containment and increases the difficulty of controlling the outbreak [1].

While diagnostic tests can be quickly developed for various diseases, they are not commonly suitable for deployment at the community and point-of-care (POC) level [3]. For example, polymerase chain reaction (PCR) is considered the gold standard in detecting an array of pathogens in humans, animals and plants [4]. Although this method is robust and accurate, performing PCR requires an expensive lab testing facility with skilled technicians. Both of which limit the test's affordability, physical accessibility, and turnaround time of results. Therefore, it is vital to innovate and develop strategies to overcome these challenges, especially for low- and middle-income countries.

An emerging technology called Loop-mediated Isothermal Amplification (LAMP) assay has been studied to be a potentially simpler and more accessible diagnostic tool than current gold standard tools such as PCR [5]. The World Health Organization described that an ideal test for controlling infectious diseases is not necessarily the most accurate one; instead, it has to be evaluated more holistically [6]. The characteristics of an ideal test include affordability by those at risk, sensitivity, specificity, user-friendliness, rapidity, and being equipment-free [6]. All these characteristics ensure that the diagnostic tests are available at POC to those at high risk, such as individuals belonging to disadvantaged backgrounds [6]. In addition, POC testing facilitates rapid diagnostics near the patient to subsequently prompt immediate yet informed health interventions.

There are various other nucleic acid amplification tests (NAAT) that use a wide array of techniques to initiate DNA synthesis and directly detect genetic material [7]. However, most of them require sophisticated instruments or elaborate methods to perform [7]. With more development and optimisation down the road, LAMP stands out to meet the criteria mentioned above.

A Brief Introduction to the LAMP Assay

LAMP is a simple single-tube NAAT that does not require thermocycling equipment to perform [8]. Instead, it only needs a simple heater block or a water bath. Unlike other NAAT methods, results can be determined through a visual colour change readout [9]. Thus, eliminating the need for confirmatory measures, such as agarose gel electrophoresis or fluorescence quantification through machines. The LAMP assay was first described in the year 2000 as a novel NAAT that amplifies small amounts (or copies) of DNA into a million copies within an hour (Figure 1) [8]. The target gene is amplified at an isothermal temperature of 60-65°C using multiple primers and a polymerase with high strand-displacing activity. The high strand-displacing activity of the polymerase aids in denaturing double-stranded DNA at lower temperatures [10].

The process requires a set of four to six primers that correspondingly bind to six to eight different regions of a target gene [8]. The binding of primers to multiple gene regions makes LAMP highly specific in pure samples. Primer sets commonly contain two outer primers (FP3 and RP3), two inner primers (FP2 and RP2), and sometimes two additional loop primers [8]. In addition to the multiple primers, another essential component of LAMP is the strand-displacing polymerase that facilitates the displacement of downstream DNA without the need for fluctuating temperatures [10].

Complementary sequences bond together, resulting in a dumbbell structure product that contains multiple sites to initiate DNA synthesis [11]. Ultimately, this process yields rapidly accumulating amplicons [11].

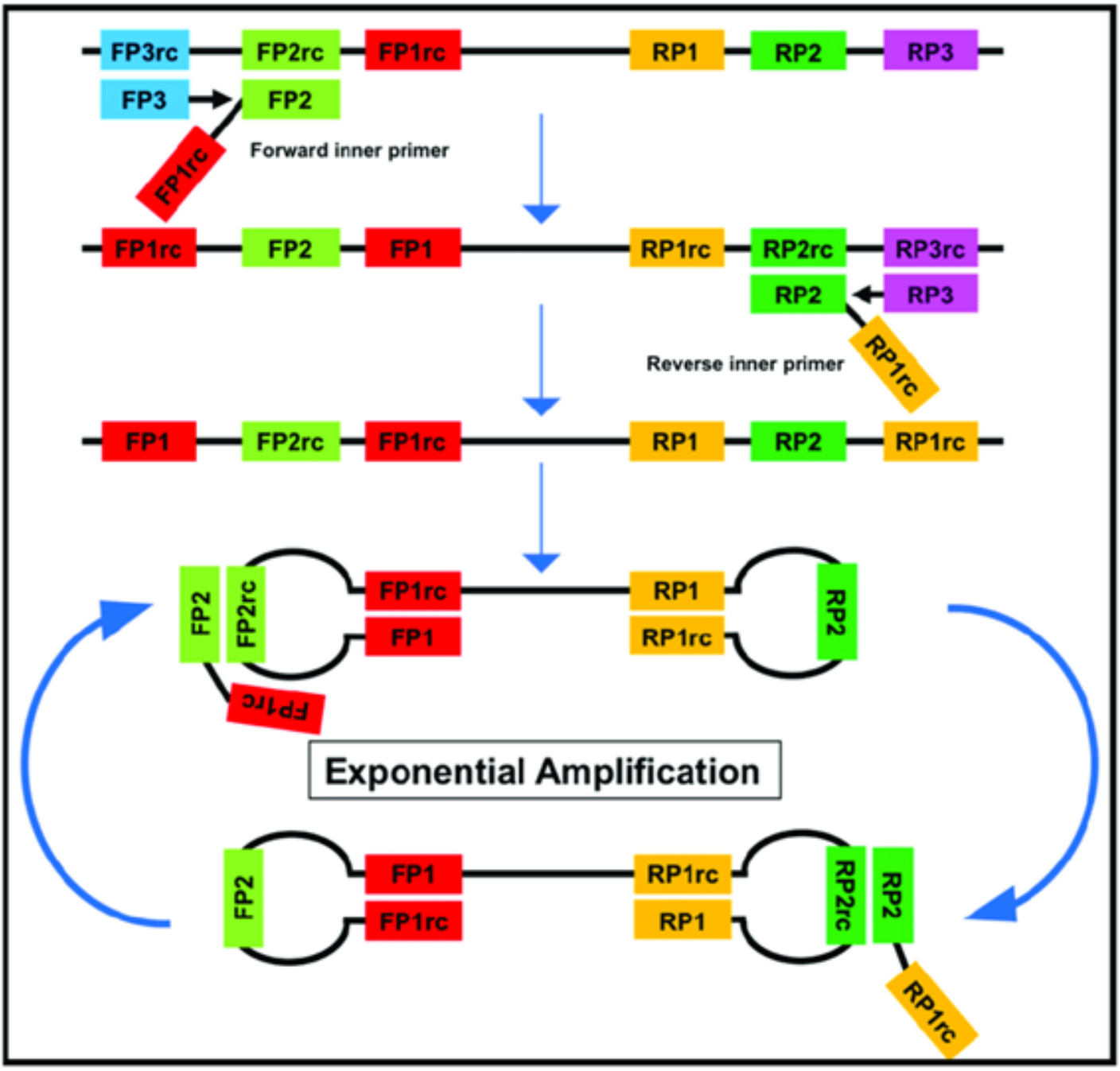

Figure 1: *Illustration of the amplification mechanism of LAMP. Amplification initiates through strand displacement by a forward inner primer. The strand-displacing activity of the polymerase helps in disrupting the double-stranded DNA, after which the reverse inner primer attaches to the newly synthesised strand. Complementary sequences bond together, resulting in a dumbbell structure product that contains multiple sites to initiate DNA synthesis. Ultimately, this process yields rapidly accumulating amplicons [11].*

LAMP is, by no means, a perfect assay. While it has high sensitivity, it also presents workflow challenges such as carryover contamination and mispriming [12, 13]. Nevertheless, researchers have been working their way towards the end of the tunnel. The method has been continuously optimised for practical applications in detecting various pathogens, primarily for providing effective POC diagnostic testing in resource-limited areas [7]. LAMP can even be adapted for a more comprehensive detection (or diagnosis) of an array of diseases in combination with other molecular approaches [14].

LAMP in COVID-19…

At the dawn of the COVID-19 pandemic, countries all over the world scrambled to realise a sudden rise in Real-Time quantitative PCR (RT-qPCR) testing demand [15]. The pandemic itself shed light on the costly laboratory set-ups, limited reagents, and a shortage of healthcare workers that contributed to a bottleneck for testing. Countries such as South Korea and New Zealand were able to keep up with the initial surge, but resource-limited and densely populated countries such as the Philippines and India struggled to keep up [15]. In an attempt to augment testing capacity for COVID-19, studies were done to adapt LAMP for SARS-CoV-2 RNA detection using an additional reverse transcription step. The modified assay called "reverse transcription LAMP" (RT-LAMP) was considered a promising POC and cost-efficient test that does not compromise accuracy [16].

To eliminate the need for skilled workers in obtaining nasopharyngeal swabs, multiple projects have successfully developed saliva testing in tandem with RT-LAMP [9, 17-22]. All these assays produced visual results in less than 30 minutes. In addition, one of these studies reported that their version of a deployable one-step RT-LAMP protocol had a specificity of >96% and sensitivity of >97% compared to the RT-qPCR and nasopharyngeal swab gold standard [23].

Currently, countries such as the USA and the Netherlands already recognise RT-LAMP as a valid alternative to RT-qPCR [24, 25]. In Austria, the Austrian Agency for Health and Food Safety (AGES) has already recommended the use of saliva-based RT-LAMP in hospitals and laboratories that have not established PCR-based diagnostics [26]. RT-LAMP's simple workflow application in COVID-19 is a leap towards achieving POC and even at-home testing. This cost-effective and rapid method may sustain routine mass testing strategies that will aid the complete reopening of the economy.

Even with the availability of vaccines, diagnostic testing remains a relevant tool in detecting "breakthrough" cases in vaccinated individuals and in testing unvaccinated people. Routine testing in a population will also determine emerging variants that may be more pathogenic. Doing so would aid in the immediate containment of these variants.

Aside from NAATs, other methods of rapid POC testing are also available such as antigen testing, but it comes with a trade-off. Although antigen tests are easily deployable and rapid, their relatively low sensitivity raises concerns among experts [15]. The test's propensity to miss infectious cases could give people a false sense of security and elicit outbreaks in countries with fewer restrictions [15]. However, it remains a valuable tool to use while other rapid yet sufficiently accurate tools are being developed [15].

...and beyond

Prior to the COVID-19 pandemic, conventional LAMP had already been utilised and developed for various infectious pathogens, such as the causative agents of pneumonia and tuberculosis [27, 28]. One of the most notable studies using LAMP was an *in vitro* study on *Trichomonas vaginalis*, the most common sexually transmitted infection in women, caused by a parasite [29]. The study found that the sensitivity of LAMP was up to 1000 times higher than the sensitivity of PCR testing. This is possibly due to the LAMP DNA polymerase's tolerance to inhibitors found in urine samples [29]. However, the LAMP test also had false-positive issues due to carryover contamination [29]. These contamination issues can be minimised through aseptic techniques or other preventive measures [13, 29].

RT-LAMP has also been used successfully in detecting other viruses such as Zika, HIV, and Ebola in recent outbreaks [30, 31]. More than plainly detecting the presence of a pathogen, RT-LAMP has the capability to distinguish pathogen subtypes. For example, a study developed a fluorescent RT-LAMP assay that could consistently detect HIV subtypes A, B, C, D, and G [32]. Furthermore, it can also be a useful POC tool to quantify HIV RNA copy numbers in low-resource, primary care, and hospital settings [32].

Surpassing human diseases, LAMP and RT-LAMP have practical applications in plants and animals, which is particularly useful for field testing in the aqua- and agriculture industries. A prime example is its use in the detection of the two most common shrimp pathogens in the Philippines: White Spot Syndrome Virus and *Vibrio* spp. [33]. Early diagnosis of disease outbreaks in shrimp populations can help farmers carry out intervening measures. Outbreaks like these could wipe out entire shrimp populations and incur significant losses on small-scale farms when not detected on time [33]. Surprisingly, the results showed that the more rapid LAMP assay was ten-fold more sensitive than conventional RT-PCR testing in detecting the White Spot Syndrome Virus [33]. On the other hand, LAMP proved to be more time- and labour-efficient in the bacterial identification of *Vibrio* spp. [33].

Similar findings apply to other pathogens such as the Batai virus in cattle and mosquitoes and the Hepatitis E virus in shellfish [34, 35]. All these demonstrate the breadth of LAMP's versatile potential to provide robust, cost-effective, and physically accessible diagnostics for various species.

Conclusion

LAMP is undergoing continuous optimisation and development with regard to disease diagnostics. Its subsequent applications demonstrated that it can be a cost-effective, physically accessible, yet sufficiently accurate alternative to RT-PCR. However, like most assays, LAMP has its drawbacks, and there is a long road ahead for improving and adapting it to detect different diseases. LAMP has always been framed as a diagnostic tool for neglected tropical diseases in low-resource settings [14]. However, the COVID-19 pandemic has exposed the vast scale of diagnostic bottlenecks beyond the developing world, and we are now seeing the first large-scale deployment of LAMP diagnostics [14]. Further optimisation of this testing strategy can build strong foundations in preparation for future outbreaks by containing the spread of pathogens. For COVID-19 and other infectious diseases, this relatively novel technology has the potential to be the LAMP at the end of the tunnel.

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CRIMINALS: MADE OR BORN?

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Insight

"Poor kid, the parents are criminals, so he will become a criminal too", "it was expectable that he would behave like this, the whole family are criminals" or "his bad genes made him do this". Those are just some common phrases that one can hear when a young person from an unstable family has chosen the wrong path. But the question is whether the environment is the culprit or whether genes decide a person's fate even before birth. People tend to justify their aggressive or antisocial behaviour by blaming their genes, but how much of it can actually be blamed on genes? Are criminals made by their environment or born due to their genetics? This article aims to shed light onto the investigation of "crime genes" and whether their existence will affect our justice system.

Maybe you have heard somebody say they behave a certain way because they inherited it from one of their parents. This is especially said for negative characteristics like criminal behaviour. But can you actually inherit the tendency to criminal behaviour, or is it just said as a defence for exhibiting behaviour that is generally not approved?

Definition of criminal behaviour

To be able to approach this question, criminal behaviour first needs to be defined: 'a phenotype within antisocial and aggressive behaviour characterised by the inability to commit to social norms' [1]. Antisocial behaviour is described as 'behaviour by a person which causes or is likely to cause harassment, alarm, or distress to persons not of the same household as the person' [2]. An antisocial personality is present in about 50-80% of imprisoned convicts, which is often associated with aggressive behaviour [3, 4]. Research mainly focused on the association between aggressive behaviour and genetics, which is why this article will mainly focus on aggressive behaviour in general, where the terms antisocial and aggressive behaviour are often used interchangeably.

Criminals from stable families

As early as 1985, researchers asked whether criminals are made or born. This implies that either the environment forms a criminal or that a person's fate is already fixed at birth due to genetics [5]. It is now widely known that social factors and environmental variables impact aggressive behaviour [6, 7]. But are there genes that ultimately lead to aggressive behaviour and thus to possible antisocial and criminal behaviour? Genetic factors seem to play a role in several psychiatric disorders, such as depression or anxiety disorders, which lead to the assumption that there is a chance that aggressive behaviour could have a genetic cause [8]. Additionally, aggressive behaviour has been essential for survival in ancient times, making it beneficial if encoded in genetics [9].

Crime gene *MAOA*

Since aggressive behaviour affects harmony and peace in a society, researchers are looking for methods to identify possibly harmful people. Therefore, scientists are looking for so-called "criminal genes" as a biomarker to identify individuals predisposed to aggressive behaviour [10, 11]. Being able to identify possible criminals early would allow the development of "anti-violence" interventions [11]. Several studies have worked on the decoding of "crime genes". One of the genes that has gotten more attention is monoamine oxidase A (*MAOA*). It encodes for the protein monoamine oxidase, which plays an important role in the metabolism of neurotransmitters. Due to its function, the enzyme is associated with regulating human behaviour, such as aggression [12-14]. Studies have pointed out that the loss of function or low activity of the enzyme (genotype *MAOA*-L) can lead to a clinical syndrome characterised by low self-control, increased impulsivity, and negative emotionality, which engages people to act aggressively and violently to minor stressors, which is why it is also called "the warrior gene" [10, 13, 15].

~~Crime~~ gene *MAOA*

However, it has been shown that only 50% of the variance in aggressive behaviour can be explained by genetics [10]. The results mentioned earlier suggest a genetic predisposition in *MAOA*-L carriers; however, some studies could not replicate the association or could only find a low correlation between the gene and aggressive behaviour [12]. In general, researchers have found evidence that genotypes cannot predefine a certain personality but rather a range of possible characteristics a person can develop. For this specific case, this would mean that not every person who carries the genotype *MAOA*-L displays aggressive behaviour [10]. However, scientific evidence has led to the assumption that the combination of biological and psychological aspects forms human behaviour [10]. This explains why people with the same genotype (e.g., *MAOA*-L) do not display the same personality. Additionally, aggression is a complex behaviour and is therefore assumed to be caused by interactions between multiple genes [13]. This is why other genes should be analysed and considered in the assessment of aggression as well [14]. In other words, the *MAOA* gene cannot be considered a "crime gene" because more factors need to be considered, such as childhood upbringing or education [10, 13].

Gene-environment interaction

The *MAOA*-L variant has been associated with aggressive behaviour in several studies; however, the influence of the environment needs to be considered. Environmental factors can interact with genes and, therefore, can either increase or decrease the genetic influence on aggressive behaviour [11]. Therefore, the presence of *MAOA*-L alone is not enough to consider a person a criminal [10, 13]. More research is needed to assess the genetic influence on behaviour,

and even more important is the correct definition and quantification of environmental factors to determine the gene-environment interactions [11]. The "social-push"-theory describes the effect of the environment on genes resulting in a specific behaviour [16]. Especially experiencing trauma, such as maltreatment, seems to be a key playing event in a person's life that leads to the development of aggressive behaviour [14]. However, there is evidence of a strong association between MAOA-L and criminal violence in offenders, but not in the average population. This means that offenders who carry this gene variant tend to be violent, whereas people who have not been criminal do not show this tendency [13].

Crime genes in the court

Knowing that there is evidence for the association between genes and aggressive behaviour and its tendency to criminal behaviour, it is not surprising that attorneys in the US and the Netherlands have been trying to justify their client's crimes with their genes. However, humanity will face an ethical dilemma in the future: if behaviour is affected by genes, how can people be made responsible for their doings? Is it ethically correct to put them in jail for something they cannot control? Are people without the "crime gene" more responsible for their doing than others [17]? On the other side, this knowledge can also be helpful in the development of "anti-violence" methods in the future, such as positive environmental conditions for people with such a genetic vulnerability for aggressive behaviour [6, 13]. Genetic counselling would also allow an assessment for a proper sentence and whether a criminal should be held criminally responsible [16].

Humans are more than a collection of genes

There are many aspects and factors to consider when answering the question as to whether criminals are made or born. However, there are indeed genes that increase the tendency to aggressive behaviour. Nevertheless, it is impossible to only blame genes for aggressive behaviour. Both genetic and social factors play a role and can act together or individually, whereby the individual role of genes, such as *MAOA*, needs to be further elucidated. It has been shown that the effect of *MAOA* seems to be dependent on the interaction with the environment [16]. So maybe the gene is not the cause for aggressive behaviour but rather the result of the environment affecting the gene expression as a result of a chain of life events.

Nevertheless, scientists fantasise about anti-criminal interventions that will be based on genetics and environmental factors in the future, which is interesting to follow. However, we must remember that a person is more than a set of genes and that social factors can influence them, making it hard to pinpoint a person's behaviour solely based on genes. Environmental and biological causes are interconnected and constantly interacting [16].

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ZEBRAS OF MEDICINE:
NON-CLASSICAL CONGENITAL ADRENAL HYPERPLASIA AND POLYCYSTIC OVARIAN SYNDROME

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Abstract

Polycystic ovarian syndrome (PCOS) and non-classical congenital adrenal hyperplasia (NC-CAH) are the most common endocrine disorders affecting females. Although the genetic predisposition and mechanism may be different, there is an overlap in the clinical presentations of symptoms, often leading to misdiagnosis. This review aims to summarise the current knowledge about the aforementioned diseases and discusses the various parameters involved in their diagnosis and treatments. PCOS and NC-CAH display similar symptoms of hyperandrogenism such as hirsutism, acne, and menstrual dysfunction. These diseases are also the leading cause of many chronic conditions in women, besides infertility, such as diabetes, cardiovascular disease, obesity, and non-alcoholic fatty liver disease. Patients diagnosed with either of these disorders are also at a higher risk for endometrial cancer and multiple pregnancy complications like miscarriages, preeclampsia, anxiety, and depression. Diagnosis usually involves testing serum levels of the altered hormone concentrations such as androgens and insulin. The existing treatments are only for managing symptoms. Endocrine disorders affect the life-long health of women and have the potential to cause multiple chronic conditions affecting the quality of life. No uniform diagnosing criteria exist, leading to the underdiagnosis or misdiagnosis of such disorders. In addition, the current tests and treatments are not curative.

Introduction

Polycystic Ovarian Syndrome (PCOS) is the most common endocrine disorder that affects 4-20% of women of reproductive age (18-44 years of age) worldwide [1, 2]. It is a multifaceted disorder with various clinical manifestations, complex pathophysiology, and unknown genetic mechanisms [3]. PCOS thereby hinders scientists and endocrinologists in understanding its aetiology and the different mechanisms involved, which are important for accurate diagnoses and treatment [4].

PCOS is also associated with multiple comorbidities such as obesity, type 2 diabetes, infertility, cardiovascular diseases, endometrial cancer, metabolic syndrome, and depression [5]. Additionally, multiple diseases exist which mimic the symptoms of PCOS, such as non-classic congenital adrenal hyperplasia (NC-CAH) and menstrual irregularities in the case of hyperprolactinemia [1]. Hence, a correct and early diagnosis is vital since the usual treatments are lifelong and involve lifestyle modifications such as exercise and diet [1].

Congenital Adrenal Hyperplasia (CAH) is a family of autosomal recessive disorders in which there is a deficiency in one of the enzymes essential for cortisol synthesis in the adrenal glands causing mild to severely impaired cortisol production [6]. The residual enzyme activity decides the severity of the disorder wherein the higher the enzyme activity, the milder the disorder [7]. Congenital Adrenal Hyperplasia consists of two types: classical - which is diagnosed in infants or neonates and non-classical or late-onset, which is usually detected in adolescence or later in life [6]. No clear distinction between the two types has been defined as the symptoms presented lie on a range of phenotypes [6]. Classical CAH is severe and can be lethal if not detected early. It usually presents itself as a salt-wasting form in the neonatal stage or genital virilisation, which only occurs in females [7]. NC-CAH is a milder form of the disorder. It is not life-threatening and usually manifests later in life or can even be asymptomatic [6]. NC-CAH is one of the most common endocrine disorders, with 0.6-9% of women being affected depending on the ethnicity [6]. It occurs more frequently in Hispanics (1:53) than in non-Hispanic people [6].

The prevalence of PCOS and NC-CAH are increasing, which, due to similar clinical manifestations (figure 1), leads to misdiagnosis and, therefore, a lack of curative treatment, making their clinical management challenging. This review aims to describe the differences between PCOS and NC-CAH in light of their cause, clinical symptoms, diagnosis, and treatment.

Cause

PCOS was first characterised by Stein and Leventhal in 1935 [1]. It is a multigenic, heterogenous disorder with no known genetic cause, even though a predisposition to it due to family history is common [4]. Recently, genome-wide association studies have identified potential genes *LHR, FSHR, INSR, ERB, THADA,* and *HMGA2* that are involved in the development of PCOS [8]. These genes are vital in pathways responsible for the production of steroids, insulin production and secretion, homeostasis, lipid metabolism, inflammation, and the hypothalamic-pituitary axis [8]. However, the genetic variants that cause PCOS are strongly influenced by epigenetic and environmental factors such as diet and lifestyle [9]. Since lifestyle varies with ethnicity, race, and population, it is difficult to make any correlations with the various genetic variants and mutations with women who have a family history of PCOS. The disruption of the hypothalamic-pituitary-ovary axis is a phenomenon that occurs during PCOS [3]. Animal studies have shown that excess prenatal androgen exposure leads to the female progeny having a PCOS-like phenotype [9]. This excess of androgen during the prenatal years results in the

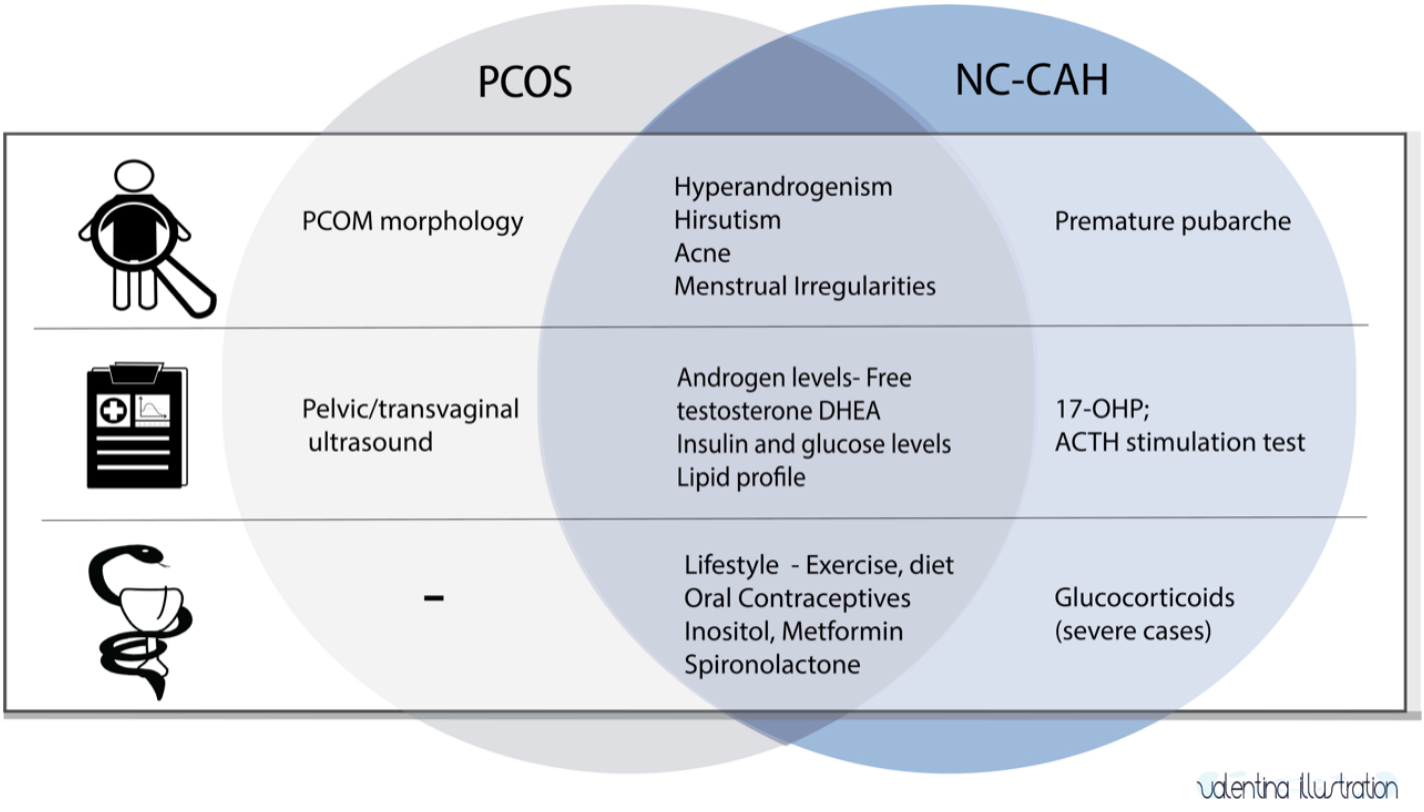

Figure 1: *Illustration depicting the similarities between PCOS and NC-CAH. These diseases have several common aspects, such as similar symptoms, diagnostic tests, and treatments. Some specific aspects in each category do exist such as testing 17-OHP levels for NC-CAH and a pelvic ultrasound for PCOS.*

hypersecretion of the luteinising hormone (LH) which eventually results in the poly-cystic morphology of the ovaries [9].

CAH is most often caused by a mutation in the *CYP21A2* gene at chromosome 6p21 [10]. *CYP21A2* encodes for the enzyme 21-hydroxylase, which is found in the adrenal glands. This enzyme converts 17-hydroxyprogesterone into 11-deoxycortisol and progesterone into 11-deoxycorticosterone and is thereby involved in the production of aldosterone and cortisol, which are responsible for sodium regulation and mental stress management, respectively [8, 11]. The *CYP21A2* gene is located next to the genes *C4* (which encodes complement 4), *RP* (serine-threonine nuclear protein kinase) and *TNX* (tenascin), forming the RCCX module [12]. However, missense mutations and non-allelic homologous recombinations in the *CYP21A2* gene result in gene conversions. In addition, during meiosis, duplications or deletions of the RCCX modules arise due to misalignment and unequal crossover [12]. These mutations on either or both of the two alleles result in the deficiency of 21-hydroxylase, which inhibits or completely blocks the conversion of 17- hydroxyprogesterone (17-OHP) to deoxycortisol and progesterone to deoxycorticosterone (steroid precursors) [6]. This leads to increased levels of 17-OHP which increases the levels of androgens giving rise to hyperandrogenism [6]. The low levels of cortisol synthesised also increase the amount of adrenocorticotropic hormone (ACTH) produced from the pituitary [12].

Clinical Presentation

There are three major features of PCOS, and most women present at least two out of three major symptoms: amenorrhea (no menstruation) or oligomenorrhea (menstruation less than six to eight times a year), increased androgen levels (hyperandrogenism), and cystic ovaries [3]. Cysts in ovaries are present in more than 70% of women affected by PCOS, along with any of the two other symptoms [2]. It has also been linked to obesity and insulin resistance leading to cardiovascular disease, infertility, type 2 diabetes mellitus metabolic syndrome, and non-alcoholic fatty liver disease [4]. These symptoms usually present themselves in adulthood, although PCOS can also affect adolescents [3].

The clinical symptoms of NC-CAH mimic PCOS. One of the main features of NC-CAH is the occurrence of premature pubarche (the first appearance of pubic hair). In a study performed on 25 females, 95% reported premature pubarche before the age of 8 [12]. However, depending on the severity of the disease, many adolescents are asymptomatic and display symptoms in late adolescence or adulthood. In post-pubarche women, the symptoms correlate to the effects of hyperandrogenism, including hirsutism, acne, androgenic alopecia (male-pattern hair loss), menstrual and ovulatory dysfunction, and infertility [13].

Diagnosis

Three guidelines with specific criteria have been outlined for the diagnosis of PCOS (figure 2) [2]. From these guidelines, the Rotterdam Criteria are commonly used to diagnose adults [2]. Complying with the Rotterdam Criteria entails diagnosing PCOS based on four phenotypes (figure 3). To investigate the ovarian morphology for the presence of PCOM (polycystic ovarian morphology), a transvaginal or pelvic ultrasound is performed to detect the string-of-pearl structure [14]. If more than 12 ovarian follicles of 2-9mm in size or an increase in the ovarian tissue are observed, the patient is diagnosed with PCOM [14].

Another major symptom of PCOS is hyperandrogenism which is categorised as either clinical or biochemical [14]. Clinical hyperandrogenism is characterised by hirsutism, while biochemical hyperandrogenism is distinguished by elevated androgen serum

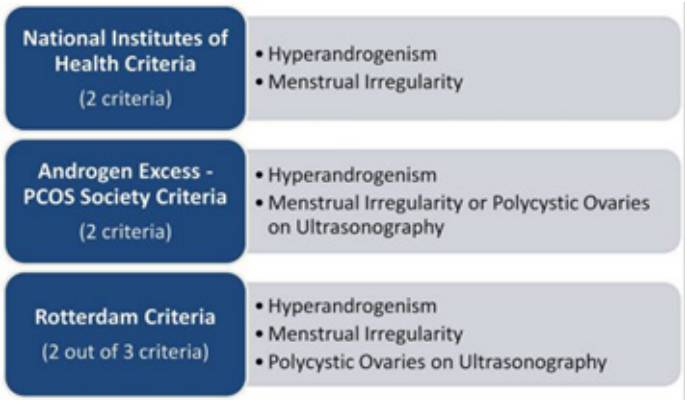

Figure 2: *Guidelines for diagnosing PCOS. Three guidelines are used worldwide for diagnosing PCOS. Each guideline has certain criteria; depending on the number of criteria the patient fulfills, it determines the diagnosis. For instance, for a patient to be diagnosed with PCOS, the patient has to meet two out of the three Rotterdam criteria [2].*

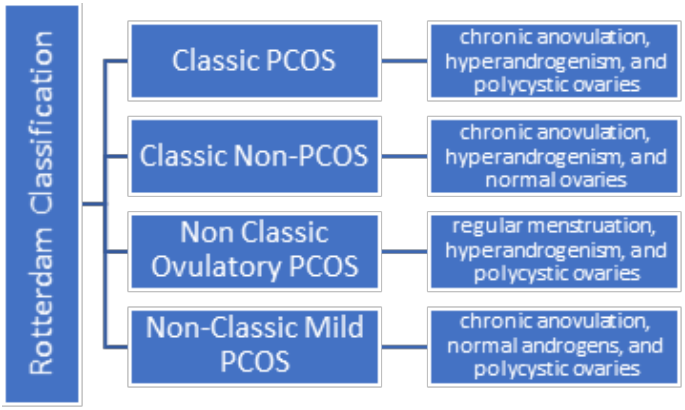

Figure 3: *Types of PCOS. Illustration displaying the different phenotypes of PCOS and the symptoms relating to each phenotype.*

levels [15]. Women who display hirsutism, acne, and androgenic alopecia are diagnosed with clinical hyperandrogenism [14]. Hirsutism is the presence of excessive coarse dark hair in women in a male-like distribution pattern such as in the inner thighs, near the navel, chest, and back. The Ferriman-Gallway score is used to define the extent of hirsutism. It is a semi-subjective system, and a score of more than 8 indicates hirsutism depending on the ethnicity of the patient [16]. For Mediterranean, Hispanic, and Middle Eastern women, a score of 9 or higher is considered hirsutism while a cut off value of 6 is used for South American women [17]. Biochemical hyperandrogenism is diagnosed when the serum levels of one or more androgens (i.e. total testosterone, free testosterone, dehydroepiandrosterone sulfate (DHEAS), and androstenedione) are elevated [15]. Blood levels of lipids, glucose, and other hormones like thyroid hormones, prolactin, anti-Mullerian hormone (AMH), and cortisol are also measured to rule out other hormone disorders that also cause menstrual irregularities such as hyperprolactinemia and thyroid disease [2].

One of the other main features of PCOS is the presence of menstrual irregularities – amenorrhea, oligomenorrhea, or inconsistent menstruation [4]. These irregularities are influenced and caused by multiple factors such as elevated androgen levels and irregular LH, follicle stimulating hormone, and oestrogen, which levels lead to ovulation issues such as anovulation (no ovulation) or oligo-ovulation (ovulating less than eight times a year) and therefore causing irregular menstruation in patients [4]. Patients are diagnosed with PCOS if they meet at least two out of the three criteria.

For NC-CAH, since the symptoms are clinically similar to PCOS, the diagnosis for hyperandrogenism is the same as PCOS, i.e. testing androgen levels and dermatological features such as acne, hirsutism, and alopecia [6]. In addition, the levels of 17-OHP and progesterone are measured, checking for their probable elevation during the pre-ovulatory phase of the menstrual cycle [12]. Furthermore, an ACTH stimulation test is performed, which stimulates the adrenal gland to produce cortisol and other steroid hormones to evaluate the concentrations of the hormones produced [18]. If the biochemical levels of these tests are borderline or give uncertain results, then genetic testing can be done to determine the genetic mutations involved, resulting in a firm diagnosis [19]. However, in developed countries, genetic testing is commonly performed, mostly in the neonatal stage, to determine the type of enzyme deficiency and its potential to be inherited by future offspring [20].

In some cases of NC-CAH, the patients may also have dysfunctional ovarian morphology, i.e. PCOM. However, diagnosing PCOS and NC-CAH is a clinical challenge, especially when the patient has non-classical PCOS with no polycystic ovaries. For such cases, measuring the 17-OHP levels and other steroid hormones after the ACTH stimulation test can aid in the differentiation of diagnoses between the two diseases [19].

Treatment

Currently, no cure exists for PCOS and NC-CAH, and present treatments are used for symptom management [19]. These treatments aim to reduce insulin resistance, decrease androgen levels, and correct anovulation [3]. One of the first-line treatments for PCOS and NC-CAH is oral contraceptive pills to lower androgen levels and regularise menstruation, thereby providing endometrial protection. Combined preparations of oestrogen and progestin have been found to decrease luteinising hormone secretions leading to a reduction in PCOM and decreasing elevated androgen levels [2].

Anti-androgenic therapy such as spironolactone is also considered for treatment. However, due to its teratogenic effect, it is not recommended [19]. The common drug administered for managing insulin resistance is metformin [21]. Recently, however, inositol, which works as a second messenger of insulin, is also recommended to manage insulin resistance [2]. Furthermore, lifestyle modifications such as regular exercise, weight loss, reduction in sugar intake, and a healthy diet with adequate protein intake is recommended for tackling obesity and has also been shown to enhance moods [4]. Specifically for NC-CAH, glucocorticoid therapy is sometimes recommended for females who are non-responsive to oral contraceptives and anti-androgen therapies [19]. Glucocorticoids suppress ACTH, thereby reducing androgen production from adrenal glands [19]. However, glucocorticoid therapy has potential side effects, is risky and, hence, is recommended only in certain cases [6].

Conclusion

Polycystic ovarian syndrome and non-classical congenital adrenal hyperplasia are common endocrine disorders that affect females. The clinical presentation of the symptoms overlaps, making their diagnosis hard, which increases the chance of misdiagnosis. Patients affected by these diseases are susceptible to additional chronic lifestyle diseases such as obesity, cardiovascular disease, non-alcoholic fatty liver disease, type 2 diabetes, and infertility. In addition,

there is an increased risk for pregnancy complications, anxiety, and depression. No cure exists for PCOS and NC-CAH, and, hence, the current treatments primarily focus on symptom management and normalisation of dysregulated hormone levels.

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INTERVIEW:
OPEN THE GATES TO OPEN SCIENCE

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Insight

Research and science are constantly changing, always evolving. One of the changes that we are going through right now is the transition towards open science. For this article three members of the Open Science Community Nijmegen, an academic organisation with the purpose of making science more transparent, rigorous, and reproducible, were interviewed [1]. I was joined by Jeanette Mostert, an associate principal lecturer at the Radboudumc, Eva Poort, a postdoctoral researcher at the Max Planck Institute for Psycholinguistics, and Jeroen Bos, an open access officer at the Radboud library. In our interview, we discussed the role of open science in (bio)medical science, and how we, as aspiring young scientists, can contribute to more open science, and the importance of preregistrations.

The what and why of open science

Let us first look at what open science actually is. Mostert says, "I think open science is very broad" "it includes many different aspects. It includes open access, it includes open data, reproducible data. So, I think the whole idea of open science is that we're making science transparent, in the way that it is conducted, in the way that it is collected, and the way that it is reported. But of course, there are also aspects like citizen science and inclusivity. So, you make science inclusive and accessible." To the question why we need open science, she answers, "Why not? I mean, it is not really a question of should we, or should we not have open science? It is more of a question of how than why." Bos agrees with this statement: "I like that view. Why not open science."

Still, the monitor of openaccess.nl reports that only 62% of articles in the Netherlands are published open access, and not all studies are preregistered [2]. What are the hurdles that need to be taken into account? "It is money, it is skills. It is the availability of resources. So, time, definitely. Experience. Tradition," says Mostert. How can we make the change to more open science?

Jeanette Mostert

Opportunities for young researchers

As (bio)medical students, we are at the start of our careers. Maybe you can remember the lectures you had about aspects of open science, but how can you bring them into practice once you start doing research? Mostert says, "I think as a young generation, you always have the responsibility to change traditions, right? Because the people who have been in the field for longer, they are so used to the way things are done. And for them, it is harder to change something. If you're new, you have to learn things anyway. So then you might just as well learn a new way of doing it instead of the old way." She gives a few practical examples: "If you are learning about the publishing system, then you might just as well learn about publishing open access. If you are learning how to write code for your analysis, you might just as well learn how to make it a reproducible code, and how to share your code. And if you are thinking about how to collect your data, you can also think about how do I collect it in a way that I can also share my data?". Poort adds, "I think it is easier for the younger generation to question the traditions because they were not there when they arose. And a lot of traditions, I think, in science are a product of how things used to be much more difficult. A lot of aspects of open science used to be a lot more difficult."

Eva Poort

Transparent statistics

One of the examples Poort gives is about making the code for your analysis more transparent by using R. By sharing your code, you make it more transparent how you did your statistical analysis, and other researchers can reproduce your study [3]. Poort says, "In terms of statistical programming, there used to be basically just SPSS, which is not very reproducible, but now it is very easy to learn about R. So, it is also much easier for the younger generation to ask, well, why are we still using SPSS? Because there is now this very easy alternative that is free and anyone can learn to use it." We first learn how to do statistics during our study. R is free, open source, useful for many types of data, and reproducible [4]. However, it can be challenging for educators to make the switch from SPSS to R. Poort knows this from her own experience when she was involved in teaching at University College London. "The statistics lecturer only knew how to use SPSS and most of the students' project supervisors also only knew how to use SPSS. So, for years they said, 'no, we cannot possibly teach the students how to do this in R because then the people who need to give them grades do not understand their statistical analyses.'". But eventually, they did change to R and now they get positive feedback from students about it.

Mostert adds another important point why young scientists should learn about open science practices: "Because if you are an established researcher it's not a big issue if you're still using SPSS. But if you are a student now, then you still have your whole career in front of you. You need to learn skills that are relevant also in the next ten years."

Preregistration

One of the practices of open science that you can already start with during your internships, is preregistration. By preregistering your study, you define your research questions, hypotheses, and plan of analysis before the start of your research [5]. In this way, you clarify what you planned to do, and show that you did not try many different analyses just to find a significant result. Moreover, Naald et al. (2020) found that the data of only 26% of animals found in study protocols, end up in a publication. They plea for preregistration to prevent reporting and publication bias [6].

I asked whether preregistration would also be a good idea for internship projects. Mostert says, "It is a great exercise, actually for the internship, because you're really forced to think about your plan in a structured way." Poort adds, "The thing that I like best about preregistration is that it really forces you to think about how you are going to do the experiment, but also how you are going to do the analysis because I have noticed in myself and in a lot of others as well. You have this idea of how you are going to do an experiment, then you do your experiment, and then you go on to the analysis, and you realize that actually, your data is not in the right format or you asked the wrong questions in your questionnaire. Then suddenly, at the end, you realise you cannot analyse your data so that you have an answer to your research question."

Preregistering your internship projects has a lot of advantages, even if you are not going to publish. "An internship is a learning experience. You can think about your study design, but then things can go wrong, right? For a million different reasons. Either your lab closes, your mice die, your cells perish," says Mostert. Later she adds, "But then you can show that you thought about your study design really well, even if nothing came out of the study. That is already something I think, maybe even more important for applying to PhD positions than having this published result."

Jeroen Bos

Practical tips for preregistration

Several websites offer opportunities for preregistering your study. Two of them are for example the Open Science Framework Registries and AsPredicted [7, 8]. Poort also gives some practical tips for preregistering your study: "The first preregistration you do is never going to be perfect. When you write the second one, you will realise, I was really not clear in the first one about what I meant to do exactly. But, as with everything in science and life in general, you always have to start somewhere and learn." She further explains that making mistakes in your preregistration is no problem at all. "An important part of preregistration is also the transparency. So even if you were not clear about something, or if you preregistered something and then you later find out that it is not actually possible to do it that way, that is not a problem. You just say in the paper: 'We made a mistake', or 'We did not realise that this was going to happen', or 'We realised we were not entirely clear in the preregistration', and you just explain what you did intend." In other words, as they formulate at Open Science Framework: "a preregistration is a plan, not a prison" [9].

Another tip by Poort is, "To try and read a lot of preregistrations of projects that are similar to yours, or at least projects that use statistical analyses and collect data similar to yours. Because then you get an idea of what they write about, and how they are going to do their data cleaning, how they are going to do their analysis, how they determine their sample size. That is how I learned to write my preregistrations. It is also a good idea to get into the habit of essentially writing them as a method section. So, then you have exactly the information that you would normally report in a paper (maybe you include a little bit more information than you would report in a paper), but that way, at least it is very clear what you are going to do, and how you plan to do it."

Her last tip is to use a template or a framework. "My first preregistration was actually using the Open Science Framework's Preregistration Challenge template. It has a long list of questions that they want you to answer, so that already gives you something to hold on to in terms of what information you need to include in your preregistration." In addition, the websites of both OSFRegistries and AsPredicted have several templates available, depending on the kind of research you are writing a preregistration for [7, 8].

Publishing Open Access

Another pillar of open science is publishing your research with open access. Open access ensures that other researchers and the general public can access your article without any barriers [10]. However, usually, there is a fee for authors if they want to publish open access. If you have the opportunity to publish as a student, Bos has a valuable tip for you: "And if you are going to publish, actually, I like to add, if you are going to successfully publish your results, there are many, many outlets for students also where you can publish in open access for free, or with heavy discount. Check the Library website for more information about open access publishing (for students)."

Conclusion

In conclusion, we discussed that open science is a very broad topic. The changes that are happening are happening slowly, and we, as future young scientists, have a part to play in the revolution towards open science. One important aspect of open science is preregistrations, as this allows us to plan our research question, hypotheses, and analysis plan before starting a project, to make research more transparent. Practical tips for preregistering your study are using a template, reading a lot of preregistrations, and learning from your mistakes every time. What will you do to make your next internship or research project more open?

Acknowledgements

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EXAM QUESTION

Question 1

In a certain inhibitory synapse, under the influence of glycine, the chloride channels are opened in the postsynaptic membrane. This causes chloride ions to flow into the cell. What effect does this have on the membrane potential?

A. Depolarisation
B. Hyperpolarisation
C. Repolarisation

(Topic from Q1 MGZ Neurology, 2020)

Question 2

A 58-year-old woman is diagnosed with a stage T3N2M0 colon carcinoma. This means that…

A. the tumour has grown into the serosa and is only present locally.
B. the tumour cells have spread to the lymph nodes as well as to other distant organs.
C. tumour cells have also been found in the lymph nodes, but no distant metastases have yet been detected.

(Topic from Q5 MGZ Immune system, 2020)

The answer to this question can be found on page 29 in this journal.

COLUMN: IT'S A BEAUTIFUL DAY TO SAVE LIVES

Guus Brand[1]

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When it was snowing and freezing earlier this year, my partner and I were out for a walk. We noticed how much we needed to mind our step on the frosty ground. Therefore, we decided to take a short trip around the block and return home via a different route. Us taking this route turned out to be a stroke of luck for someone else. As we made our way home, I faintly heard a voice calling out for help, and we turned around. Hunched up against a wall, sitting in a pile of snow and ice, was an elderly woman. She slipped, fell, and had difficulties getting up. She told us she had been there for about half an hour, calling out for help, but no one seemed to have heard her. As she was sitting in the snow out in the freezing cold, her temperature seemed to have dropped, and she was in a tremendous amount of pain. At least, that was my assessment even though she kept saying we did not need to stay because she did not want to burden us. When we realized she could not get up by herself, my medical mind began to race.

Chaotically, I tried to memorize and execute the emergency medical protocol, but I kept skipping parts and getting back to them later. We hoisted the woman onto a chair and tried to keep her warm, but I quickly realized she had probably broken her hip and needed professional medical attention. I ran to the nearest general practitioner (GP) office. On the way there, I realized I should have called an ambulance and did so while running back, without ever reaching the GP office. The emergency paramedics arrived on the scene within minutes, and I was thoroughly impressed with how they managed to get a grasp on the situation, assess the patient's health and background, and decide what needed to happen next while they were busy getting her in the ambulance. Within minutes they were gone, driving off to the nearest hospital, and I was sure never to hear from that sweet elderly lady again.

For me, the story above signifies a couple of things, but mainly how much respect I have for emergency medical care workers. In the UK, emergency departments are required to decide whether to admit or discharge a patient within four hours after arriving at the Emergency Department (ED) [1]. This means that within this limited time frame, doctors, nurses, and paramedics are supposed to treat acute life-threatening-injuries, get familiar with the situation, diagnose, and then make a decision that could seriously impact a patient's life. A book written by Paul Brand, my dad, calls every patient contact and consultation a dance [2]. If this applies to the ED as well, it should be regarded as a tango or a quickstep.

To add to this acute time pressure, not all patients in the ED are sweet elderly ladies who happened to slip and break their hip. Not all are thankful to be cared for, and certainly not all make a full recovery while in the ED. Working in an ED means being involved in a lot of human suffering and being exposed to death frequently. Patients may be verbally or physically aggressive and may resist care. All this can go on throughout the day. A recent review published in *Nature* found that one in five emergency care workers in the USA and Canada meet diagnostic criteria for PTSD, and this does not even take the COVID-19 pandemic into account [3].

I would like to take this opportunity to say thank you to all emergency department medical staff. You do fantastic work, and I have the utmost respect for your profession. It is my firm belief that you should be put in the spotlight more often. However, I do not envy you and feel anxious to start my clinical rotation in the ED because I think it would be hard for me to let a patient go and not know how they fared. Luckily for me, my elderly patient of more than ninety years old showed up on my doorstep just a few months later with a bouquet to thank us. She was almost fully recovered from her hip surgery and could walk without assistence.

Reaction

In his column, Guus Brand brings up a few topics. First, the quick, efficient and high-quality care that was provided by the paramedics in the prehospital situation. They take care of ill or injured patients, sometimes under difficult circumstances. Not only difficult because of the severity of injuries, but sometimes they have to deal with the aggression of patients or bystanders. Therefore, I support the message of Brand for being grateful for the work the paramedics are doing in the field.

Second, Brand referred to a study about the prevalence of PTSD in ED staff pre-COVID pandemic. This study was performed in the USA with a different medical health care system than the Netherlands, but also in the Netherlands, the workload in the emergency medical care is experienced as high. This feeling has grown in the current situation of the pandemic. Many health care professionals who deal with COVID-19 patients feel exhausted. The demands are high due to the high number of patients with COVID-19 while there is shortage of staff.

In the first wave of the pandemic, we experienced a great challenge. However, we took on the job as a team and felt a lot of support. This made that we could perform at our best. We have experienced that this support was of help. That is why I appreciate the thankfulness that Guus Brand shows in his last paragraph. Let us be thankful and give our support to everyone who needs this at the moment. You can think of medical staff, police, teachers but also your favourite bar or restaurant or whoever you think needs your support.

Wouldn't it be beautiful if we all say "Thank you!" to someone tomorrow?

Jeroen Veldhuis
Emergency Physician, Isala Hospital Zwolle

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PATHOGENS IN PERMAFROST: THE NEXT PANDEMIC?

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Insight

'Russia anthrax outbreak affects dozens in north Siberia' (BBC news) [1]; 'The permafrost pandemic: could the melting Arctic release a deadly disease?' (Greenpeace) [3]; 'Deep Frozen Arctic Microbes Are Waking Up' (Scientific American) [4]; 'All hell breaks loose as the tundra thaws' (The Guardian) [5]; 'How thawing permafrost could resurrect long-dormant diseases' (Arctic Today) [6].

These are just some of the headlines published in the last few years regarding thawing permafrost and the potentially pathogenic microorganisms it contains. While these scenarios might once have seemed like the plot of a semi-interesting apocalypse movie, the COVID-19 pandemic and the unusually large 2021 wildfires raging across the Arctic circle have suddenly made worrying about thawing permafrost releasing some unknown ancient disease much more rational. But, can these diseases still form a threat to us? And if so, how is this possible? This editorial will give a brief overview of the current state of microorganisms in permafrost and their potential to cause the next big outbreak.

For the third summer in a row, the coldest region of Russia was on fire. In Yakutia, in the Northeast of Siberia, one of the hottest and driest summers was recorded in over a 100 years with temperatures up to 38 °C. According to the Guardian, the wildfires destroyed a record-breaking 18.16 million hectares, which is over four times the size of the Netherlands. For the first time in recorded history, the smoke reached the north pole 3000 kilometres away, darkening the snow and ice and causing it to melt faster due to increased heat absorption from the sun [7, 8].

It is not unusual to have some summer fires in this region, and they actually support the ecosystem's health, but the summer of 2021 has proven to be exceptional [9, 10]. In addition to the fires releasing about 970 megatons of carbon dioxide into the atmosphere - twice as much as was released in the previous year - the relentless heat also accelerates thawing of the Siberian permafrost, partly due to increased microbial activity [9, 11]. Besides storing enormous amounts of methane and carbon, which contribute to global warming upon release, permafrost also contains a whole zoo's worth of frozen microorganisms [12]. This raises the question: should we be worried about the microorganisms preserved in these frozen areas causing the next pandemic?

Permafrost

Before we get to this, let us dive into what permafrost exactly is. Classically, permafrost is defined as soil that has remained frozen for at least two years in a row, but it can be millions of years old [11]. About 25% of the land on Earth is underlain by permafrost [11]. Overlaying this permafrost is the so-called 'active layer', which is subject to seasonal freeze-thaw cycles. Therefore, melting of a few centimetres up to several meters of permafrost is normal. However, due to global warming, the depth of the active layer has been increasing, resulting in a decrease of the permanent permafrost [13]. This is where thawing permafrost becomes problematic. Permafrost has accumulated organic matter, such as remnants from plants and animals, over many, many years. When temperatures rise, microorganisms that were once frozen can be reactivated and start decomposing these remnants (figure 1). As a result of this, carbon dioxide (CO_2) and methane (CH_4) are released; these gasses could possibly accelerate global warming and, therefore, even more thawing of permafrost [11, 14]. This is already happening: while in the last 100 years, the global average temperature increased by 0.7 °C, the average temperatures of the upper layer of Arctic permafrost increased by 3.0 °C, a phenomenon known as 'Arctic Amplification' [15]. According to one projection, this could lead to a 90% reduction of near-surface permafrost by 2100 [16]. Theoretically, thawing of the permafrost could then also release reawakened historical pathogens that might pose a threat for future outbreaks [17, 18].

Surviving the cold

It seems quite impossible for any organism to remain viable after being frozen for hundreds to thousands of years. However, microorganisms have developed several strategies that enable them to survive in temperatures below 0 °C. One of these is to enter a dormant state, where there is low metabolic activity and production of specialised proteins that help the cell survive [19]. In 2007, researchers found bacteria that survived frozen conditions for 25,000 years [20]. Another recent example is a study where an Acanthamoeba was used as 'bait' to isolate a large, viable DNA virus from a 30,000-year-old permafrost layer [21]. The microorganisms do not just survive but are shown to be metabolically active and grow at temperatures up to -20 °C [22]. Even when investigating the oldest ice layers on Earth of approximately 34-million-years old, viable metabolically active bacteria were found that had about eight million birthdays to their name [23].

And it is not just all geriatric pathogens chilling out in the guts of long-dead mammoths [24]. We, as humans, are also regularly depositing new microorganisms on the ice. One study performed on the Kahiltna Glacier in Alaska, a popular climbing spot, calculated that climbers annually produced over two megatons of human waste [25]. Besides the faeces leading to highly unhygienic situations that resulted in diarrhoea for almost a third of all 132 interviewed climbers, the bacteria in the faeces also turned out to be able to survive for an

Permafrost can not only be found on our planet, but also on Mars, Jupiter, Saturn, Uranium, Neptune and Pluto. Intriguingly, if microorganisms are surviving in the Earth's permafrost, there might also be cellular life on the coldest planets of our Solar System [2].

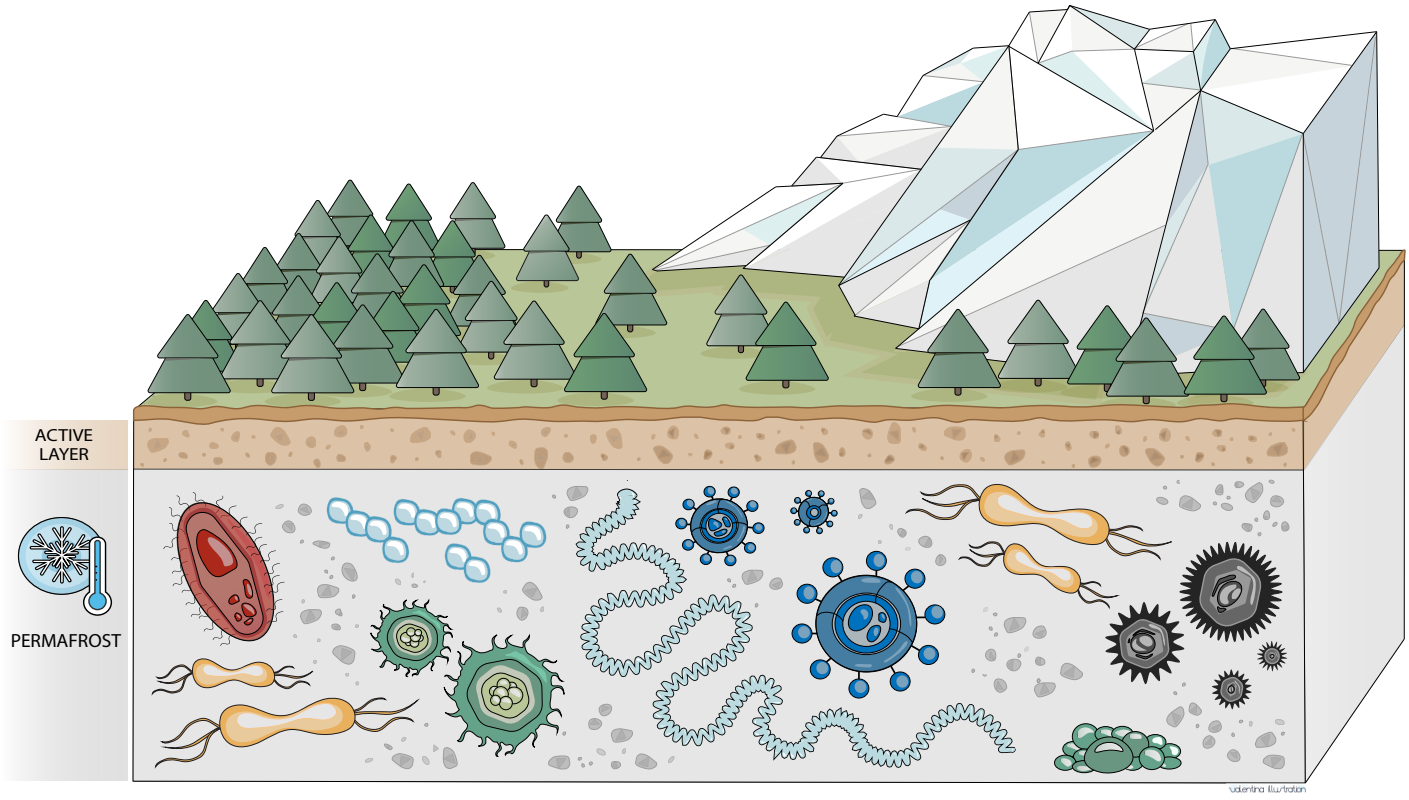

Figure 1: *A simplified image of the active layer and the micro-organisms in permafrost (not to scale).*

extended period of time and were predicted to travel through the glacier resurfacing at lower, more accessible, elevations [25]. Thus, both old and new microorganisms can be found in permafrost.

A real threat?

In practice, dangerous, living pathogens caused a large anthrax outbreak in 2016 in Arctic Russian Siberia [18]. This resulted not only in the untimely demise of over 2000 reindeer but also in the hospitalisation of 20 people and eventually the tragic death of a 12-year old child [18]. The victims were infected by spores of *Bacillus anthracis* that had been preserved in frozen soil for over 75 years [18]. Experts believe that spores were released from previously frozen burial grounds of infected carcasses that surfaced due to high thawing rates [26]. As there are up to 13,885 cattle burial grounds in Russia alone, primarily because of anthrax outbreaks, re-emerging anthrax provides a real threat to those living nearby [27].

Following this concept, similar outbreaks could occur due to other robust pathogens. One of the more infamous candidates may be the variola virus, which causes smallpox. Before this devastating disease was eradicated in 1980, it is estimated to have been the cause of 10% of all deaths worldwide during the last millennium [28]. Smallpox vaccination campaigns were abolished decades ago, increasing the fraction of the world's population that has no immunity against smallpox. Combine this with the rise in immunosuppressed individuals, and smallpox suddenly reappears as a significant threat [29].

Already in 1991, when a grave filled with 19th century mummified smallpox victims were found, the Russian authorities were worried that floods might wash the virus to inhabited regions [30]. A similar burial ground was unearthed in 2004 when French and Russian researchers found a wooden grave in the permafrost containing five mummies [31]. Samples from this uncanny discovery revealed that the most likely cause of death was variola infection. The researchers could link this particular strain to the smallpox epidemic over 300 years earlier in 1714 [31].

Fortunately, until now, no viable virus has ever been isolated from a mummified smallpox victim. However, researchers do not entirely exclude the possibility that there may be viable variola viruses buried somewhere in the Arctic [32]. It is a Schrödinger's cat situation: as long as we cannot find it, it might be alive, and it might be dead.

Additional danger can be found in the potential for permafrost to act as a reservoir for antibiotic resistance genes. Using PCR techniques, researchers have found resistance genes for antibiotics such as beta-lactams and tetracycline, that possibly spread to these areas by migrating birds and airborne bacteria [33, 34]. In a world where antibiotic resistance is one of the top ten global health problems, such a reservoir periodically releasing resistance genes may become problematic [35].

This 'will they, won't they' seems to be a recurring trope when researching this topic; there might be a viable and devastating disease hiding in this Pandora's box of microorganisms, but there might also be none. Some researchers argue that the freeze-thaw cycles necessary to incorporate microorganisms into the permafrost as well as the subsequent thaw and release are likely to inactivate microorganisms [36, 37]. In addition, permafrost soil is generally acidic, limiting survival [34, 37]. Even the previously described Siberian anthrax outbreak – often invoked to emphasize the dangers of frozen pathogens – is now questioned to be solely caused by permafrost thawing; the discontinued reindeer vaccinations and increased reindeer numbers are very likely to have contributed as well [37].

Resurrecting viruses

There may be more danger in scientists resurrecting viruses from the viral genomic material that is preserved. For example, researchers previously managed to recover the entire viral genome of the 1918 Spanish flu, which was responsible for over 50 million deaths [38]. While this does provide essential information on, for example, the prevention and control of future pandemics - which is relevant for obvious reasons - certain dangers are attached. In 2014, researchers recovered the viral genome of two unknown viruses from 700-year old caribou faeces, one of which was able to infect plants under laboratory conditions [39]. Since there is not much known about the infectivity of these viruses, this kind of research must be done with great care for safety if we want to avoid 'pathogens escaping from the lab' scenarios [34].

Conclusion

So, in the context of thawing permafrost, pandemics are not likely to be one of the major problems. There are plenty of other issues to worry about regarding warming and thawing of permafrost soil, such as increased intensity and frequency of wildfires; infrastructure collapse due to damage to roads, buildings, pipelines and houses; coastal erosion and flooding; poleward disease-vector spread of, for example, ticks; and let us also not forget permafrost stores up to 1580 billion megatons of organic carbon, almost twice as much as is present in the entire atmosphere [14, 36, 37]. Gradual emissions of these thawing stocks into the atmosphere will likely have significant effects on global warming.

However, it never hurts to be safe on the health side. We simply do not know the extent of the danger yet, and more research is necessary to determine what microorganisms in permafrost are actually viable and able to cause an outbreak upon release. Especially since climate change, 'last chance'-tourism, and increased oil, gas, and mineral extraction in the Arctic circle lead to higher levels of direct human contact with thawing permafrost, monitoring and surveillance of the permafrost and its microorganisms might not be a luxury [40, 41]. We are already dealing with one pandemic. Let us not make it more!

Acknowledgements

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CORRECT ANSWERS TO THE EXAM QUESTIONS

Answer question 1:
B. Hyperpolarisation

Chloride ions are negatively charged. When these negative ions flow into the cell, the membrane potential will become even more negative than the resting membrane potential as more negative charge will be inside the cell compared to outside the cell. When this happens, we talk about hyperpolarisation of the membrane potential. The hyperpolarisation will cause an inhibitory effect as more positive charge is needed to depolarise the cell. Glycine is one of the major inhibitory neurotransmitters.

For further reading:
Siegel, A., Sapru, H. Chapter 7: Neurotransmitters in Essential Neuroscience, 4th edition (Wolters Kluwer, Philadelphia, 2019).
Siegel, A., Sapru, H. Chapter 6: Synaptic Transmission in Essential Neuroscience, 4th edition (Wolters Kluwer, Philadelphia, 2019).

During the exam, 44% of the participants answered this question correctly.

The exam questions can be found back on page 24 in this journal.

Answer question 2:
C. tumour cells have also been found in the lymph nodes, but no distant metastases have yet been detected.

Colon carcinomas can be staged by the use of the TNM-classification. T3 stands for tumour invasion into the subserosa, N2 shows that there are metastases in four or more regional lymph nodes, and M0 indicates that there are no distant metastases. Therefore, tumour cells have been found in the lymph nodes, but not in other distant organs.

For further reading:
Tanis P. J., Beets-Tan R. G. H., Marijnen C. A. M., Nagtegaal I. D., Punt C. J. A. Chapter 10: Tumoren van dunne en dikke darm in Leerboek oncologie, 9th edition (Bohn Stafleu van Loghum, Houten, 2017)

Akkoca, A. N., Yanık, S., Ozdemir, Z. T., Cihan, F. G., Sayar, S., Cincin, T. G., Cam, A., & Ozer, C. (2014). TNM and Modified Dukes staging along with the demographic characteristics of patients with colorectal carcinoma. *International journal of clinical and experimental medicine*, 7(9), 2828–2835.

During the exam, 96% of the participants answered this question correctly.

RECENT HIGH-IMPACT PAPERS FROM RADBOUDUMC RESEARCHERS

Aster Witvliet[1]

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 – published by researchers from the Radboudumc – will be discussed.

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Investigating protein-protein interactions with a new proximity labelling technique

When investigating the function of a protein, it can be incredibly helpful to know which other proteins interact with your protein of interest. Information on what other kinds of proteins are found in its neighbourhood can, for example, be a starting point for studying the function of a protein of interest. Proximity biotinylating is a powerful technique that allows identification of protein-protein interactions *in vivo*. In this technique, a proximity biotinylating enzyme, e.g., BioID, is fused to the protein of interest using a CRISPR-Cas9 mediated knock-in or a plasmid-based transfection. When biotin is added to cells containing this construct, proteins proximal to the protein of interest are biotinylated by the proximity biotinylating enzyme. The biotinylated proteins can then be isolated and subsequently analysed using mass spectrometry. However, a major drawback of this technique is that it can only be performed in cells that can be genetically engineered. To overcome this limitation, Santos-Barriopedro *et al*. published an article in *Nature communications* (impact factor = 14.9) in which they developed a modified proximity biotinylating technique that relies on a recombinant enzyme called ProtA-Turbo [2]. ProtA-Turbo consists of a proximity biotinylating enzyme fused to Protein A. Protein A allows for localisation of the recombinant enzyme to an antibody targeting the protein of interest. The authors show the functionality of ProtA-Turbo working together with multiple antibodies against both proteins and histone modifications. In the demonstration of their methods, they identified FLYWCH1 as an unreported proximal protein for the H3K9me3 histone modification ($p<0.05$). This technique is applicable in any cell type and thus allows for the identification of proteins located closely to any protein or histone modification for which an antibody is available.

When is gallbladder removal beneficial?

In about 5% of people with gallstones, complications can arise, such as intense pain in the abdomen, nausea, and vomiting. A common treatment for these symptomatic patients is a cholecystectomy, which entails the surgical removal of the gallbladder. However, many patients who undergo this surgery still experience pain afterwards, including some patients where pain persists and does not subside. To improve decision-making in treatment for patients with gallstone associated pain symptoms, Latenstein *et al*. analysed data from two multicentre prospective trials to identify predictors of pain reduction after cholecystectomy, which was reported in *JAMA Surgery* (impact factor = 14.8) [3]. Patients were evaluated for pain symptoms six months after their cholecystectomy. The study identified seven factors that could be used as predictors of clinically relevant pain reduction. Clinically relevant pain reduction was found with higher frequency in patients who were older, had no previous abdominal surgery, had a high pain score, and had pain radiating towards the back. Patients with clinically relevant pain reduction were also shown to report pain reduction with simple analgesics, nausea during pain attacks, and no heartburn (C statistic = 0.8, 95% CI 0.74-0.84). In the future, surgeons could use these predictors to assess the likelihood of pain reduction after cholecystectomy in their patients. In this way, the results from this study might help contribute to a reduction in cholecystectomies without pain relief and thus prevent patients from having redundant surgeries.

Dysregulation of autophagy impairs neuronal development

Koolen-de Vries syndrome (KdVS) is a rare neurodevelopmental disorder that has been linked to heterozygous loss of function mutations in the *KANSL-1* gene. This gene is likely important in the regulation of autophagy, a process in which the cell degrades its own components. A study by Linda *et al*. published in *Autophagy* (impact factor = 16.0) aimed to better understand how *KANSL-1* mutations affect neuronal function in KdVS patients by using iNeurons, which are neurons derived from human induced pluripotent stem cells (iPSCs) [4]. iNeurons from KdVS patients were found to have less functional synapses than healthy controls and showed a pattern of neuronal signalling associated with immature networks. As *KANSL-1* had previously been shown to be relevant for autophagy regulation, dysregulation of autophagy was further pursued as the potential cause of KdVS neuronal dysfunction. When using a marker for autophagosomes and immunofluorescence microscopy, it was found that KdVS neurons had a much higher amount of autophagosomes. To explain why this accumulation of autophagosomes occurred, the authors investigated differentially expressed genes in KdVS and found that expression of SOD1, an important antioxidant enzyme, was decreased. Due to a deficiency in SOD1, reactive oxygen species (ROS) levels were much higher in KdVS cells, which in turn induced the accumulation of autophagosomes. To further confirm this theory, iNeurons were treated with aponycin, a drug that inhibits ROS production. Upon treatment with aponycin, KdVS iNeurons had lowered ROS levels and reduced autophagosome accumulation ($p<0.01$, $p<0.005$). Furthermore, when iPSCs were treated with aponycin during differentiation towards iNeurons, functional synapse numbers and neuronal signalling resembled that of healthy control cells. Taken together, these findings indicate that the increased ROS and autophagosome accumulation found in KdVS patient neurons interfere with neuronal development. Therefore, antioxidants like aponycin, which rescued the KdVS phenotype in iNeurons, could be an interesting possibility for treatment of KdVS patients. Moreover, mutations in *KANSL-1* have been linked to Parkinson's disease in a genome wide association study, meaning that the findings from this paper might help contribute to increased understanding not only of KdVS, but potentially also of Parkinson's disease.

References

1. Radboudumc. "Onze impact in 2020" *Impact 2020* (2020). https://www.radboudumc.nl/over-het-radboudumc/impact-2020
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3. Latenstein, C.S.S., *et al.* A Clinical Decision Tool for Selection of Patients With Symptomatic Cholelithiasis for Cholecystectomy Based on Reduction of Pain and a Pain-Free State Following Surgery. *JAMA Surg*, e213706 (2021).
4. Linda, K., *et al.* Imbalanced autophagy causes synaptic deficits in a human model for neurodevelopmental disorders. *Autophagy*, 1-20 (2021).

RAMS

A Word from the Board of RAMS

Dear reader,

Thank you for taking the time to read the 21st edition of RAMS. This is the first edition of the eighth board, and although the planning was tight, we can say that we delivered an edition to be proud of. Therefore, I want to thank everyone that contributed to this edition. As chair, I am happy with the work we delivered, and it excites me to see what we will accomplish during the rest of the year. I hope that you have learned something new while reading and you enjoyed going through the edition as much as I did.

As of now, the first semester of the academic year has almost passed, and the first round of exams are done. I hope that all of you have had the opportunity to enjoy on-campus lectures again. I am sure that you have missed them and your fellow students as much as we have. With everyone back on campus again, we also had the opportunity to distribute the edition at different spots on campus. To bring the RAMS edition to you in this way has brought us great joy. I hope future editions will also find their way to you.

Don't forget to check the other RAMS activities and see you soon!

On behalf of the eighth board of RAMS,

Jarno Baars
Chair of the eighth board of RAMS 2021-2022

General Board

RAMS is directed by the general board, which consists of four (bio)medical students. As members of the board, they frequently meet to make sure all activities run smoothly. Moreover, they are in close contact with the supervisory board and the editorial staff. If you have any questions on general, promotional, or financial subjects, please contact the general board of RAMS via voorzitter.rams@ru.nl.

Editorial Board

The editorial board, which consists of three (bio)medical students, is responsible for the contents of the journal, from reviewing the submitted papers to their rejection or publication. Furthermore, the editorial board is in charge of writing editorials and determining the general layout. For questions concerning the content of the journal, please contact the editorial staff via hoofdredactie.rams@ru.nl. To submit papers, consult the 'for authors'-section on our website or mail to submit.rams@ru.nl.

Reviewers

Reviewers have been trained with the help of masterclasses given by professors and teachers at Radboud university medical center. With their knowledge, the reviewers are able to judge the submitted scientific and editorial articles.

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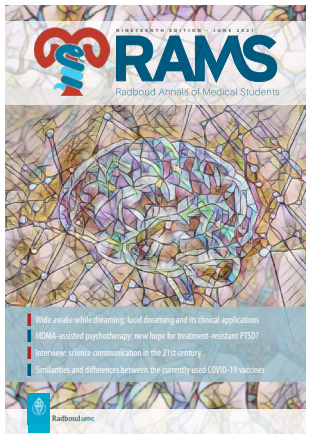

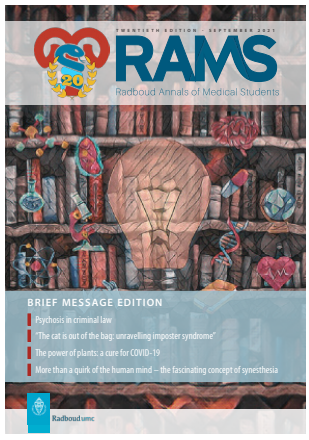

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