SEVENTEENTH EDITION - DECEMBER 2020

RAMS

Radboud Annals of Medical Students

Radboudumc

COLOPHON

Edition	Seventeen
Date of publication	December 2020
Place of publication	Nijmegen

CONTACT
89 MFVN - Radboud Annals of Medical Students
Geert Grooteplein Noord 21
6525 EZ Nijmegen
Delivery code: M230.01.106
ramsresearch.nl

EDITORIAL BOARD

Kavita Lips	*Chairwoman*
Mèlanie Reijnaers	*Scientific*
Yfke Prins	*Editorial*

GENERAL BOARD

Lotte van der Net	*Chairwoman*
Laura Hooijmaijers	*Vice-Chair*
Hajar Rotbi	*Treasurer*
Guusje van Veldhuizen	*Public Relations*

EDITORS
Quentin Marsman
Natalie Ludwig
Elisah Geertman
Femke van Hout

CHIEF DESIGN
Heidi Schigt

ILLUSTRATOR
Heidi Schigt *heididesign.nl*

PHOTOGRAPHER
Davíd van Groeninghen

SUPERVISORY BOARD
ir. Tjeerd van den Broek
mr. Bob de Jonge
dr. Jur Koksma
dr. Dirk Schubert
Stijn van der Velden (Student Assessor Medical Faculty)

All authors and their supervisors granted written permission for publication.

Scientific articles have blue accents. Editorial articles have red accents.

Copyright © 2020 RAMS. All rights reserved.

FROM THE EDITORIAL BOARD

Dear readers,

First of all, I want to say that you have done an impressive job in the past few months during the COVID-19 outbreak and that I am incredibly proud of you. Even though the virus is intangible and not concrete for many of us, it has caused immense stress and undermined our psychological well-being. Many of you might have been confronted with sickness, job loss, disappointment or death. The situation may have impacted your ability to be productive and engage in your everyday activities, and it may even have made you stop doing the things you love. It is an understatement to say that the coronavirus has caused significant disruption to life as we knew it. Not only the virus itself, but also the negativity it causes has been highly contagious.

Nevertheless, despite all these circumstances, you managed to find the time and effort to pick up this journal and read about science. As you might have already heard a billion times this year, engagement in science is more important than ever. Being your new Scientific Editor-in-Chief, I could not agree more. We live in a society where we seem to have a love-hate relationship with science and technology. The continuous change in scientific understanding can be challenging; it can even evoke fear. It is my aim and RAMS' aim, to encourage you to develop your own intrinsic motivation to read and write about science. We, as the board of RAMS, hope to expand and deepen your knowledge by exposing you to various intriguing topics written by our beloved editors or submitted by curious students like you!

In the 17th edition of our journal, we will discuss various topics concerning immunology ranging from the implementation of bacteriophages to dyslipidemia and hyperglycemia in psoriatic inpatients. We will reveal whether you unconsciously choose your partner based on their smell, to create diversity in MHC-genes, as well as explain the impact of viral infections on the development of multiple sclerosis. If you have seen the movie "Brain on Fire" you might recognise the topic of our Zebra of Medicine, which discusses anti-NMDA encephalitis, normally associated with psychiatric disorders; here we describe a putative alternative aetiology. This and many more articles can be found in our first edition as the new board of RAMS.

We hope that you enjoy this edition as much as we do, that it inspires and motivates you, and that it increases your enthusiasm for science and research! Above all, we want to wish you, your loved ones, and your fellow students a safe passage through this challenging time.

Yours faithfully,

Mèlanie Reijnaers, Scientific Editor-In-Chief

INDEX

A NEW CLASS OF ANTIBIOTICS: TYPE III SECRETION SYSTEM (T3SS) VIRULENCE BLOCKERS

Maider Echeveste[1]

[1] MSc Biology, Radboud university Faculty of Science, Nijmegen, the Netherlands, in collaboration with Department of Crop and Soil Sciences, Colorado State University, Fort Collins, CO, USA

Abstract

Background: Although antibiotics have saved innumerable lives, due to the disproportionate use of antibiotics for the treatment of humans and farm animals, multidrug resistance (MDR) has emerged. Innovative strategies to tackle antibiotic resistance that allow for a more sustainable therapy to treat bacterial infectious diseases are urgently required.

Objective: This literature review examines the inhibition of conserved growth-dispensable type III secretion system (T3SS) as promising virulence blocking strategy.

Methods: T3SS blockers were categorised according to the inhibitor-screening detection method used to identify them, which were the whole cell-based high-throughput screens or the target-based-inhibition approach. These methods allowed us to perform a comprehensive examination on methodology cross-validation.

Results: Caminoside-A, derived from a marine sponge extract, was the only compound identified via both screening approaches, indicating that this is a very promising candidate.

Conclusion: T3SS has been shown to be an attractive target for the development of anti-bacterial drugs. Further smart and refined inhibitor search designs will improve virulence blocker discovery. Future advances on T3SS inhibition are expected to circumvent the increasing problems derived from MDR.

KEYWORDS: type three secretion system (T3SS), virulence blocker, inhibitor screening

Antibiotic therapy is the most commonly used strategy to overcome bacterial pathogenic infections. Still, the steady misuse of antibiotics has led to multidrug-resistance (MDR), which has recently spread and exacerbated, causing the rise of global resistance. Antibiotics kill bacteria by affecting their key cellular processes, translating in a strong selective pressure to develop resistance against antibiotics [1]. Antibiotics cause a lot of collateral damage as they target not only bacterial pathogens but also resident flora [1]. The decreasing effectiveness of antibiotics is, thus, gradually leading us to a post-antibiotic era.

Although MDR is now one of the top three threats to global public health, the current entry rate of new antibiotics is very low [2]. The lack of novel antibiotic targets, the absence of high throughput screen (HTS)-induced host cell toxicity, and the disinterest of pharmaceuticals to invest in the discovery of long-term solutions are some of the factors causing this novelty shortage. Since, HTS allows us to conduct millions of parallel chemical tests to assess for their inhibition potential of new compounds, this method is auspicious. Conversely, MDR has, unfortunately, given an impulse to shift to more expensive and broad-spectrum antibiotics [3].

Current anti-bacterial therapies

Extension of the post-antibiotic era is highly dependent on the introduction of novel anti-bacterial medicines. Here, narrow-spectrum or even organism-specific antibiotics form a particularly exciting new alternative direction. This includes success stories such as the top-selling narrow-spectrum lipopeptide daptomycin (marketed as Cubicin®) antibiotic [1]. Advances in the field of the so-called "antibiotic stewardship" also promote to stay optimistic with the use of traditional single-agent antibiotics. Furthermore, knowledge on the survival advantage of biofilms has led to promising studies. Interestingly, currently in use, low molecular weight antibiotics were shown to be prone to penetrate and kill species-specific biofilms (e.g., rifampicin) [2]. Other remarkable options include antibiotic adjuvants, which either block resistance or boost host response and monoclonal antibodies or modifiers of the immune system [1].

Disarming bacterial pathogens: virulence blockers

Long term solutions to the challenge of antibiotic resistance are essential. The latest studies on bacterial virulence factors and toxins have allowed us to understand how pathogenic bacteria can manipulate diverse host cellular processes [4]. Upon encountering a human host, they use a variety of mechanisms to either hinder or evade host defences. Bacterial components that interact with the host include capsules, lipopolysaccharides, exo- and endotoxins, invasins, and adhesins [5].

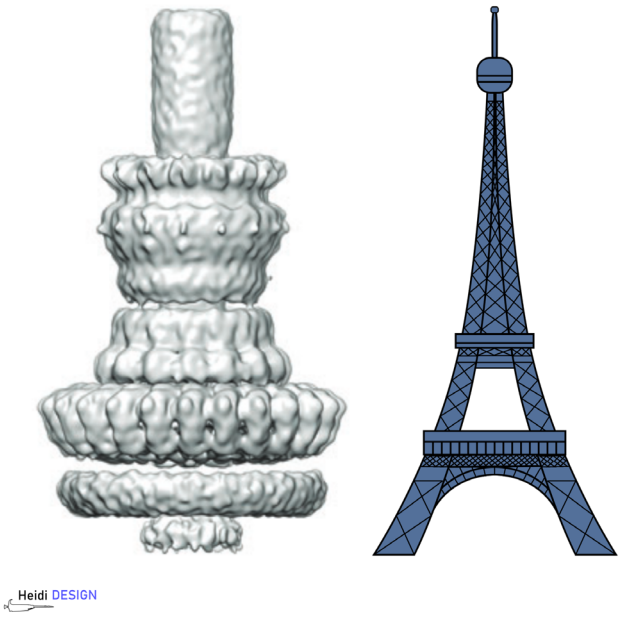


Figure 1: Similarities between type III secretion system and the Eiffel Tower
Left image adapted from Figure 1 of Galan et al. (2014). Surface view of the 3D reconstruction of the cryo-EM map of the Salmonella typhimurium needle complex13. Danger signal represents the hazard for host cells of having an effector protein introduced into their body.

For effective host-microbe interaction, some toxins or effector proteins require microinjection into target cells. Found in many pathogenic gram-negative bacteria, the type III secretion system (T3SS) constitutes one type of sophisticated secretion system. Examples of well-studied T3SS-carrying bacteria encompass *Chlamydia trachomatis*, enteropathogenic or enterohemorrhagic *Escherichia coli* (EPEC/EHEC, respectively), *Pseudomonas aeruginosa*, *Salmonella enterica*, and *Shigella* or *Yersinia* spp. [6]. Back in 1991, preliminary studies in *Yersinia* spp. virulent protein secretion already hinted the existence of T3SS, illustrated in *Salmonella* spp. [7-10]. T3SS specialised effector proteins, thus, allow for many gram-negative bacteria to induce the establishment of infection both in mammals, fish, insects, or plants [11]. Actually, T3SS could form a diversity of weapons that disrupt host homeostasis and immune defences via the disruption of cell cytoskeleton; cell trafficking; epithelium integrity; cell death/survival pathways; inflammatory signalling, or specific adaptive immunity triggering [12].

In this light, small organic molecules targeting T3SS virulence or T3SS-specific virulence blockers could target the virulence factor without affecting bacterial growth. This would translate into a chemical virulence attenuation strategy that would ensure low selection pressure for the emergence of resistant strains. Considering the conservation of T3SS across several bacterial pathogens, the discovery of effective broad-spectrum T3SSs would be ground-breaking. Furthermore, host commensal flora does not use virulence mechanisms and will, therefore, not be targeted [3]. Molecular docking of atomic structures with 3D-cryo EM allows us to observe bacterial injectisome components with remarkable high-resolution [13, 14]. This detailed view aids in virulence blocker search. It could be argued that mutations leading to resistance produce changes on the T3SS *per se*. However, mutations affecting T3SS have shown to lead, with few exceptions, to avirulent phenotypes [15].

T3SS: general architecture and mode of action
T3SS injectisome is a multiprotein complex that transports effector proteins directly from the bacterial cytosol into eukaryotic host cells. The membrane-embedded export apparatus controls the secretion of proteins and anchors the apparatus to the bacterial membrane [13]. The ATPase allows recognition of the chaperone/effector complex as well as provides energy to insert and unfold effector proteins into the apparatus. Partially unfolded effector proteins are secreted through a needle at the extracellular channel (Figure 1) [3].

The needle complex is composed of a base structure embedded in the bacterial envelope and a needle-shaped extension that protrudes from the bacterial surface. On the one hand, the base is formed by two inner (IR1/2) and two outer membrane rings (OR1/2) mediated by a neck [13]. On top, the tip assures regulation of the secretion and forms a scaffold for the pore-forming unit in the host cell (translocon) assembly [3]. The most widely conserved injectisome components have homologues in bacterial flagella, which drive cell motility. In this sense, non-flagellar T3SS (NF-T3SS) structures, like secretin at the outer membrane, form optimal inhibitors (Figure 2) [13].

Ideal requisites for virulence blocker design
Unaffected bacterial growth is a *sine qua non* (without which, not) for optimal anti-T3SS candidate compound screening. Both *in vitro* (e.g., HeLa cells) and *in vivo* (murine/mammal models) tests must confirm that compounds are non-toxic for the host, the cells of the human body. Pharmacokinetics and -dynamics studies should assure that bacterial cell permeability and drug efflux pump hurdles are overcome. Moreover, T3SS-blockers must be NF-T3SS specific. Finally, structure-activity relationship evaluation will allow identification of the biological active functional groups of T3SS compounds and synthesis of more efficient drugs. This last step includes conducting a successful clinical trial.

Research aims and approach

- Summarise currently described T3SS virulence blockers according to the inhibitor screening detection method used to identify them, which are whole cell-based (WCB) HTSs or target-based (TB)-inhibition [6];
- identify double positives for both screening-methods allowing to perform a comprehensive examination on methodology cross-validation;
- highlight promising future directions for T3SS research.

Compounds detected via both approaches might appear to be the most promising virulence blockers. We hypothesise that the combination may counterbalance the deficiencies found on each of the approaches.

Methods

The division of this review into two screening approaches was inspired by the screening categorisation of Charro & Mota *et al.* [6]. Our review performs an updated and refined search of their general scheme, while looking to highlight positive cross-methodology compounds. The literature search was mainly conducted via PubMed and Academic Google Scholar. Query search included one or more combinations of key-words: "type three secretions system," "virulence blocker," "inhibitor screening," "effector protein," "transcription," "atomic characterization," "needle tip," and "ATPase." After screening for the earliest reviews, key experimental studies were filtered based on their abstracts. Upon filtering, experimental investigations on WCB-HTS and TB-approach-related papers were narrowed down to 19 and 37, respectively. Reference researches were eventually studied in detail, with a special focus on the methodology employed. All relevant articles found up to May 30th, 2019, were included.

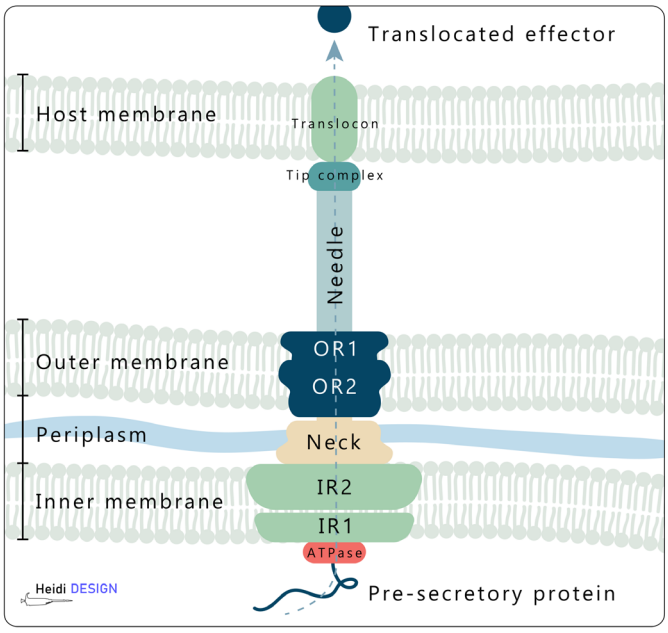


Figure 2: Schematic representation of type III secretion system of gram-negative bacteria

IR1/2: Internal ring 1 and 2; OR1/2: Outer ring 1 and 2; PG: peptidoglycan; C: chaperones.

Results

Whole cell-based, high throughput screening

Both for natural and synthetic compounds, WCB-HTS has been the primary approach for the study of T3SS virulence blockers. Considering the particular study design, this category has been further subdivided into three main read-outs (ROs).

RO1: Gene expression regulation of T3SS genes

Studies on the classic example of MDR and biological warfare strain, *Yersinia* spp., allowed the first detailed description of T3SS inhibitors discovered via WCB-HTS. These studies delivered the still extensively investigated presumed virulence blockers: salicylidene acylhydrazides (SAHs). In this study, the effector protein promoter of *Yersinia* spp. (Yop) was fused with *lux*AB, which encodes for a luciferase, to detect T3SS gene expression/inhibition (Figure 3A) [16]. Follow-up studies on SAHs allowed to characterise and confirm its inhibitory effects on other T3SS-carrying pathogenic bacterial genera, including *C. trachomatis*, *S. enterica*, and *S. flexneri* [17-20]. *S. flexneri*-T3SS vs SAHs elucidating research speculated that the detrimental effects of SAHs on the bacteria's T3SS could potentially be linked to needle assembly [19].

Notwithstanding SAHs promising discovery, their study design had some clear disadvantages [16]. The luciferase light reporter may have been suppressed at different levels: directly by selective binding to the luciferase enzyme causing its inhibition, by bacterial toxicity or, by blocking transcription factors and interfering on *lux*AB expression. Moreover, the bacterium secretes a negative regulator until it attaches to a eukaryotic cell. Thereby, Yop effector measurement is biased.

Subsequent experiments using SAHs compounds screened from this first WCB study overcame these observed limitations. Candidate inhibitors proved to specifically inhibit Yop as well as its translation after infection of HeLa cells [15]. Another pioneering study identified other non-SAHs *Yersinia* spp. T3SS inhibitors, using the same reporter gene: lux operon-luminescence [21]. Under low Ca^{2+}, *Yersinia* spp. bacterial growth is inhibited while T3SS expression is boosted. Taking advantage of this peculiar growth under low Ca^{2+}, luminescence was used to measure bacterial growth and T3SS inhibition. This approach was able to reject compounds that act indirectly on the inhibition of secretion by the toxic effects on bacteria [21].

T3SS-carrying phytopathogens studies are highly representative of the mode of action of T3SS-carrying human pathogens. Plants make use of phenolic compounds to combat the bacterial invasion. Studies on *Dickeya dadantii* showed how specific phenolic compounds-o-coumaric acid (OCA) and t-cinnamic acid (TCA) could upregulate phytopathogens' T3SS [22]. Furthermore, these discoveries are permitted to identify other potential T3SS repressors based on OCA and TCA isomers/analogues-like p-couramic acid, which is an isomer of OCA [23]. More recently, the T3SS of *Ralstonia solanacearum* was described to be induced by plant oleanic and ferulic acids [24, 25]. Investigations in phytopathogen *Erwinia amylovora* corroborated the broad T3SS inhibition capacity of SAHs as well as other virulence factors. Also, iron-scavenging showed to be fundamental in protecting bacteria from SAHs [26]. The transcriptional fusion of effector protein of *P. aeruginosa* with the GFP-encoding gene allowed us to screen a library of phenolic compounds as a reverse strategy to look for novel T3SS inhibitors. Since *P. aeruginosa* infects both humans and plants, plants may also include T3SS in their plant defence system (Figure 3A) [27].

RO2: *In vitro* T3SS effector protein absence/presence

HTS is performed to detect the absence/presence of the T3SS inhibitor derived effector protein, by using an enzyme-linked immunosorbent assay and using an anti-T3SS substrate (Figure 3B). Caminoside-A, a compound able to inhibit secreted proteins from specific bacteria, was the first T3SS inhibitor discovered when using this experimental approach. This particular screening design confirmed that growth was not inhibited [28]. Subsequent investigations in *Salmonella* spp. in the same study design detected two fungal analogues or compounds: Cytosporone B and fusaric acid [29, 30]. Both compounds allowed bacterial growth and showed no toxic effect on human cells. More studies on fusaric acid inferred that T3SS inhibition might be performed via effector protein (SicA) chaperone blockage [31].

RO3: T3SS secreted or translocated reporter proteins

Another WCB study subjected *Salmonella* spp. to HTS of small molecule libraries of natural and synthetic compounds. For that, based on a reporter construct of the effector (SipA) fused to the *Yersinia* phospholipase YplA, protein secretion *in vitro* was measured (Figure 3C). Upon cleavage of the supplied substrate, secreted SipA resulted in measurable fluorescence. This way, T3SS inhibitor 2-imino-5-arylidene thiazolidinedione was detected, which was subsequently demonstrated to also block *Yersinia* spp. T3SS [32]. Based on translational fusion of the effector protein with mature TEM-1-lactamase, translocation can be directly detected in living host cells, using fluorescent lactamase substrate. The application of this HTS allowed us to identify six *Yersinia* spp. specific translocation effector inhibitors (Figure 3C) [33].

Target-based inhibitor design

TB inhibitor design includes the rational design of inhibitors impeding the assembly of specific structures/effectors of T3SS (Figure 3). Inhibitors screening for T3SS components could be performed via rationally design WCB-HTS targeting specific T3SS components or HTS aided by structure-specific computer docking experiments.

Translocators of T3SS-(poly)peptides and proteins

In an attempt to isolate *Salmonella* spp. SipB effector protein inhibitors, in the form of exogenous purified polypeptides or polypeptides to

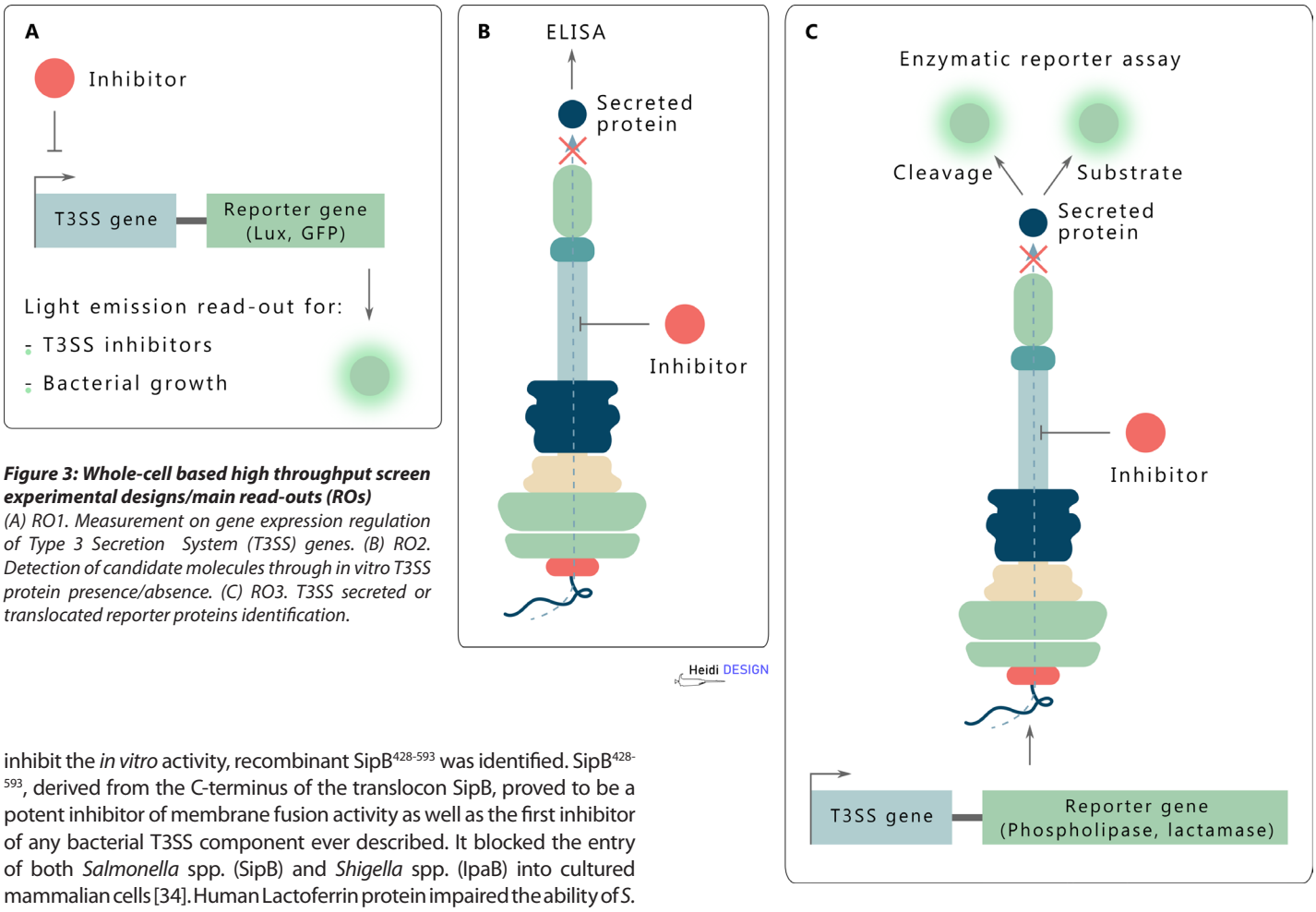


Figure 3: Whole-cell based high throughput screen experimental designs/main read-outs (ROs)
(A) RO1. Measurement on gene expression regulation of Type 3 Secretion System (T3SS) genes. (B) RO2. Detection of candidate molecules through in vitro T3SS protein presence/absence. (C) RO3. T3SS secreted or translocated reporter proteins identification.

inhibit the *in vitro* activity, recombinant SipB$^{428\text{-}593}$ was identified. SipB$^{428\text{-}593}$, derived from the C-terminus of the translocon SipB, proved to be a potent inhibitor of membrane fusion activity as well as the first inhibitor of any bacterial T3SS component ever described. It blocked the entry of both *Salmonella* spp. (SipB) and *Shigella* spp. (IpaB) into cultured mammalian cells [34]. Human Lactoferrin protein impaired the ability of *S. flexneri* to infect HeLa cells by inducing the degradation of its translocon proteins (IpaA and IpaB) [35]. More recently, CoilA and CoilB, 15 amino acid long peptides presenting coiled-coil regions of the translocon of EPEC (EspA), effectively inhibited EPEC T3SS-dependent hemolysis of red blood and mammalian cell invasion via EspA translocon blockage. Later translocation secretion studies on EHEC and *Citrobacter rodentium* reinforced and expanded CoilA and CoilB protection ability [36].

ATPase

As a highly conserved cytosolic protein, ATPase is an attractive target for broad-applicability anti-T3SS drugs [13]. Three prospective inhibitors were successfully identified via a computational screening of commercially available drug-like molecules against the active site of *Y. pestis* ATPase, followed by *in vitro* structure characterisation [37]. These three candidates were also effective against *Burkholderia mallei* together with *S. flexneri* ATPases, BsaS, and Spa47, respectively [37, 38]. Targeting ATPase might lead to concerns because of cross-reactivity with human enzymes. However, it has been argued that bacterial enzymes have less than 25% of identity to human ATPases and are significantly different, especially at the active sites [37].

AraC like transcription factor

AraC transcription factors (TFs) belong to the multiple adaptational response protein family. Inhibition of AraC-mediated activation of transcription of key virulence genes (including T3SS) has proven to be a promising and effective strategy. The screening of small molecules libraries for species-specific AraC-like TF *in vitro* aided to narrow down some candidate inhibitors. Benzimidazole, SE-1, and regacin were identified to prevent species-specific AraC-like TF and DNA binding in *Yersinia pseudotuberculosis* (LcrF), *S. flexneri* (VirF), and *C. rodentium* (RegA), respectively [39-41]. Benzimidazole and regacin had its efficacy further corroborated/ filtered *in vivo* [39, 41].

Effector protein

Chemical modifications and experimental screening of derivates of thiohydrazones, thiohydrazides, or oxamine acids allowed us to identify a potential chlamydial T3SS effector inhibitor: CL-55. CL-55 included all the requisites targeted: low host toxicity, highly selective with T3SS, good pharmacokinetic properties, and simple synthesis scheme. It inhibited chlamydial injection *in vitro* and selectively blocked the translocation of its effector protein, IncA [42]. A follow-up study successfully tested its application in a mice model [43]. Additional CL-55 *in vitro* assays and mice models both in *S. enterica* and *P. aeruginosa* corroborated its broad applicability [44, 45].

Needle tip complex

While it often remains enigmatic in which the T3SS component is inhibited, MBX2359, a class of phenooxyacetamide, is a notable exception. MBX2359 has shown to specifically target *P. aeruginosa* needle tip. Here, *P. aeruginosa* T3SS inhibitor-resistant mutant was used, and those mutation sites were then located via thorough sequencing [46]. This first study was further expanded by testing the efficacy of MBX2359 in a mouse model where *P. aeruginosa* abscess formation was alleviated, and the immune clearance of the mouse was boosted [47]. Computational modelling of *Salmonella* typhi SipD invasive needle tip protein combined with molecular docking experiments resulted in the detection of Caminoside-A. Interestingly, Caminoside-A matches the T3SS inhibitor described from a marine sponge 15 years ago via WCB-HTS [48].

Discussion

Overcoming limitations of WCB-HTS

The most pressing concern in the field of virulence blockers is the identification and characterisation of specific targets. Thereby, WCB phenotypic HTS are inherently biased and should be carefully evaluated to avoid false positives. SAHs are the most widely studied chemical compounds, all derived from WCB-HTS. Different studies have shown that SAHs mode of action could result in a synergistic effect. Observed T3SS inhibition could arise from diverse unspecific targets. These include other key conserved bacterial proteins/enzymes, iron chelation, heme metabolism pathways, or basic bacterial physiology, including flagellar T3SS-mediated swimming motility [19, 49-53]. In order to cope with these limitations, one study successfully chemically optimised SAHs and described more selective blockers [54]. In order to determine where T3SS inhibitors interrupt the T3SS process exactly, more general standardised orthogonal assays for HTS have been alternatively proposed [55]. In this regard, quantitative structure-activity relationship analysis, including statistical molecular design, forms a promising refined approach for the evaluation and smart search of putative T3SS inhibitors [56].

The good and the bad of TB approaches

TB is potentially superior to the phenotypic screens as the search is rational and directed against a very specific target. Thus, the TB approach translates into typically faster, cheaper, and more efficient inhibitors. In fact, they can be identified using less experimental screening and potentially lower off-target hits. Still, detected compound activity is often inactive on the cellular level and may lack cell permeability. For instance, some candidate TB approach derived T3SS ATPase and AraC-like TF inhibitors either have limited solubility or show non-specific inhibition at higher concentrations, respectively [37, 40]. Thus, in practical terms, not being useful for therapeutic applications. Here, state-of-the-art rational *in silico* design seems like a prospective bet, especially considering the major advances in the knowledge of the atomic structure of several injectisome components and the rapidly evolving computational docking experiments [13, 14]. Moreover, most T3SS proteins lack enzymatic activity, and this further hampers HTS.

Caminoside-A: positive on methodology cross-validation

Caminoside-A has been the only compound identified via both screening approaches WCB-HTS and TB inhibitor design. It constitutes a promising virulence blocker having proved its functionality in a real biological system (EPEC), together with its specific interaction with *S. enterica* needle tip complex, SipD [28, 48]. In other words, this ensures that uncertainties found in one approach are counterbalanced on the other.

Current status and next steps

The refinement of presented inhibitor design approaches, WCB and TB, would already allow for more advanced and encouraging studies. In this regard, developing cell-free systems to properly assess and screen for T3SS inhibitor *in vitro* is detrimental [6]. Still, further and clinic widespread use would face unique obstacles. For example, T3SS inhibitors do not affect bacterial replication outside the host. Thus, the standard minimum inhibitory concentration measurement will not be useful for assaying comparative drug activity. Instead, an effective dose of inhibitor will require animal models to predict appropriate dosing for humans [57]. Since most T3SS target a specific subset of pathogenic bacteria, accurate and fast diagnosis will be a prerequisite for treatment. For this purpose, point-of-care devices are currently developed [58].

Conclusion and outlook

T3SS is an essential and conserved virulence factor dispensable for bacterial growth. This review has illustrated that T3SS can also be viewed as an attractive target for the development of anti-bacterial drugs. Caminoside-A has been identified for the first time, to the best of our knowledge, as the only compound positive both for WCB and TB approaches. Critical steps for the near future should include the identification of the mode of action of known T3SS virulence blockers and *in silico* rational design of new T3SS inhibitors. These discoveries are expected to circumvent the increasing problems derived from MDR. Conversely, new drugs targeting T3SS of multiple pathogenic gram-negative bacteria will allow for a more sustainable therapeutic/prophylactic pathogen protection.

Acknowledgements

RAMS would like to thank Marien de Jonge, PhD, Radboudumc, Nijmegen, The Netherlands, for providing the author of this article with feedback. This is a shortened version of the original literature thesis. Therefore, detailed descriptions of pathogenic bacterial diseases have been excluded. For the extended version, please contact the corresponding author, Maider J. Echeveste (M.Echeveste@science.ru.nl).

References

1. Laxminarayan, R., *et al.* Antibiotic resistance-the need for global solutions. *Lancet Infect Dis* **13**, 1057-1098 (2013).
2. Pendleton, J.N., *et al.* Clinical relevance of the ESKAPE pathogens. *Expert Rev Anti Infect Ther* **11**, 297-308 (2013).
3. Mcshan, A.C. & De Guzman, R.N. The bacterial type III secretion system as a target for developing new antibiotics. *Chemical biology & drug design* **85**, 30-42 (2015).
4. Keyser, P., *et al.* Virulence blockers as alternatives to antibiotics: type III secretion inhibitors against Gram-negative bacteria. *J Intern Med* **264**, 17-29 (2008).
5. Wilson, J.W., *et al.* Mechanisms of bacterial pathogenicity. *Postgrad Med J* **78**, 216-224 (2002).
6. Charro, N. & Mota, L.J. Approaches targeting the type III secretion system to treat or prevent bacterial infections. *Expert Opin Drug Discov* **10**, 373-387 (2015).
7. Michiels, T. & Cornelis, G.R. Secretion of hybrid proteins by the Yersinia Yop export system. *J Bacteriol* **173**, 1677-1685 (1991).
8. Rosqvist, R., *et al.* Intracellular targeting of the Yersinia YopE cytotoxin in mammalian cells induces actin microfilament disruption. *Infect Immun* **59**, 4562-4569 (1991).
9. Kubori, T., *et al.* Supramolecular structure of the Salmonella typhimurium type III protein secretion system. *Science* **280**, 602-605 (1998).
10. Blocker, A., *et al.* The tripartite type III secreton of Shigella flexneri inserts IpaB and IpaC into host membranes. *The Journal of cell biology* **147**, 683-693 (1999).
11. Cornelis, G.R. & Van Gijsegem, F. Assembly and function of type III secretory systems. *Annu Rev Microbiol* **54**, 735-774 (2000).
12. Pinaud, L., *et al.* Host Cell Targeting by Enteropathogenic Bacteria T3SS Effectors. *Trends Microbiol* **26**, 266-283 (2018).
13. Galan, J.E., *et al.* Bacterial type III secretion systems: specialized nanomachines for protein delivery into target cells. *Annu Rev Microbiol* **68**, 415-438 (2014).
14. Schraidt, O., *et al.* Topology and Organization of the Salmonella typhimurium Type III Secretion Needle Complex Components. *PLOS Pathogens* **6**, e1000824 (2010).
15. Nordfelth, R., *et al.* Small-molecule inhibitors specifically targeting type III secretion. *Infect Immun* **73**, 3104-3114 (2005).
16. Kauppi, A.M., *et al.* Targeting bacterial virulence: inhibitors of type III secretion in Yersinia. *Chem Biol* **10**, 241-249 (2003).
17. Muschiol, S., *et al.* A small-molecule inhibitor of type III secretion inhibits different stages of the infectious cycle of

Chlamydia trachomatis. *Proc Natl Acad Sci U S A* **103**, 14566-14571 (2006).
18. Negrea, A.*, et al.* Salicylidene acylhydrazides that affect type III protein secretion in Salmonella enterica serovar typhimurium. *Antimicrob Agents Chemother* **51**, 2867-2876 (2007).
19. Veenendaal, A.K.*, et al.* Small-molecule type III secretion system inhibitors block assembly of the Shigella type III secreton. *J Bacteriol* **191**, 563-570 (2009).
20. Tree, J.J.*, et al.* Characterization of the effects of salicylidene acylhydrazide compounds on type III secretion in Escherichia coli O157:H7. *Infect Immun* **77**, 4209-4220 (2009).
21. Pan, N.J.*, et al.* Targeting type III secretion in Yersinia pestis. *Antimicrob Agents Chemother* **53**, 385-392 (2009).
22. Yang, S.*, et al.* Type III secretion system genes of Dickeya dadantii 3937 are induced by plant phenolic acids. *PLoS One* **3**, e2973 (2008).
23. Li, Y.*, et al.* The plant phenolic compound p-coumaric acid represses gene expression in the Dickeya dadantii type III secretion system. *Applied and environmental microbiology* **75**, 1223-1228 (2009).
24. Wu, D.*, et al.* Oleanolic Acid Induces the Type III Secretion System of Ralstonia solanacearum. *Frontiers in microbiology* **6**, 1466-1466 (2015).
25. Zhang, Y.*, et al.* Ferulic Acid, But Not All Hydroxycinnamic Acids, Is a Novel T3SS Inducer of Ralstonia solanacearum and Promotes Its Infection Process in Host Plants under Hydroponic Condition. *Frontiers in plant science* **8**, 1595-1595 (2017).
26. Yang, F.*, et al.* Small-molecule inhibitors suppress the expression of both type III secretion and amylovoran biosynthesis genes in Erwinia amylovora. *Mol Plant Pathol* **15**, 44-57 (2014).
27. Yamazaki, A.*, et al.* Derivatives of plant phenolic compound affect the type III secretion system of Pseudomonas aeruginosa via a GacS-GacA two-component signal transduction system. *Antimicrob Agents Chemother* **56**, 36-43 (2012).
28. Linington, R.G.*, et al.* Caminoside A, an antimicrobial glycolipid isolated from the marine sponge Caminus sphaeroconia. *Org Lett* **4**, 4089-4092 (2002).
29. Li, J.*, et al.* Cytosporone B, an inhibitor of the type III secretion system of Salmonella enterica serovar Typhimurium. *Antimicrob Agents Chemother* **57**, 2191-2198 (2013).
30. Li, J.*, et al.* Fusaric acid modulates Type Three Secretion System of Salmonella enterica serovar Typhimurium. *Biochem Biophys Res Commun* **449**, 455-459 (2014).
31. Li, J.-Q.*, et al.* The role of the LRRK2 gene in Parkinsonism. *Molecular Neurodegeneration* **9**, 47 (2014).
32. Felise, H.B.*, et al.* An inhibitor of gram-negative bacterial virulence protein secretion. *Cell Host Microbe* **4**, 325-336 (2008).
33. Harmon, D.E.*, et al.* Identification and characterization of small-molecule inhibitors of Yop translocation in Yersinia pseudotuberculosis. *Antimicrobial agents and chemotherapy* **54**, 3241-3254 (2010).
34. Hayward, R.D.*, et al.* A Salmonella SipB-derived polypeptide blocks the 'trigger' mechanism of bacterial entry into eukaryotic cells. *Mol Microbiol* **45**, 1715-1727 (2002).
35. Gomez, H.F.*, et al.* Human lactoferrin impairs virulence of Shigella flexneri. *J Infect Dis* **187**, 87-95 (2003).
36. Larzabal, M.*, et al.* Effect of coiled-coil peptides on the function of the type III secretion system-dependent activity of enterohemorragic Escherichia coli O157:H7 and Citrobacter rodentium. *Int J Med Microbiol* **303**, 9-15 (2013).
37. Swietnicki, W.*, et al.* Identification of small-molecule inhibitors of Yersinia pestis Type III secretion system YscN ATPase. *PLoS One* **6**, e19716 (2011).
38. Case, H.B.*, et al.* Shutting Down Shigella Secretion: Characterizing Small Molecule Type Three Secretion System ATPase Inhibitors. *Biochemistry* **57**, 6906-6916 (2018).
39. Garrity-Ryan, L.K.*, et al.* Small molecule inhibitors of LcrF, a Yersinia pseudotuberculosis transcription factor, attenuate virulence and limit infection in a murine pneumonia model. *Infect Immun* **78**, 4683-4690 (2010).
40. Koppolu, V.*, et al.* Small-molecule inhibitor of the Shigella flexneri master virulence regulator VirF. *Infect Immun* **81**, 4220-4231 (2013).
41. Yang, J.*, et al.* Disarming bacterial virulence through chemical inhibition of the DNA binding domain of an AraC-like transcriptional activator protein. *J Biol Chem* **288**, 31115-31126 (2013).
42. Zigangirova, N.A.*, et al.* Development of Chlamydial Type III Secretion System Inhibitors for Suppression of Acute and Chronic Forms of Chlamydial Infection. *Acta naturae* **4**, 87-97 (2012).
43. Koroleva, E.A.*, et al.* Small molecule inhibitor of type three secretion suppresses acute and chronic Chlamydia trachomatis infection in a novel urogenital Chlamydia model. *Biomed Res Int* **2015**, 484853 (2015).
44. Nesterenko, L.N.*, et al.* A small-molecule compound belonging to a class of 2,4-disubstituted 1,3,4-thiadiazine-5-ones suppresses Salmonella infection in vivo. *J Antibiot (Tokyo)* **69**, 422-427 (2016).
45. Sheremet, A.B.*, et al.* Small Molecule Inhibitor of Type Three Secretion System Belonging to a Class 2,4-disubstituted-4H-[1,3,4]-thiadiazine-5-ones Improves Survival and Decreases Bacterial Loads in an Airway Pseudomonas aeruginosa Infection in Mice. *Biomed Res Int* **2018**, 5810767 (2018).
46. Bowlin, N.O.*, et al.* Mutations in the Pseudomonas aeruginosa needle protein gene pscF confer resistance to phenoxyacetamide inhibitors of the type III secretion system. *Antimicrob Agents Chemother* **58**, 2211-2220 (2014).
47. Berube, B.J.*, et al.* Impact of Type III Secretion Effectors and of Phenoxyacetamide Inhibitors of Type III Secretion on Abscess Formation in a Mouse Model of Pseudomonas aeruginosa Infection. *Antimicrob Agents Chemother* **61**(2017).
48. Samykannu, G.*, et al.* Investigations of binding mode insight in Salmonella typhi type-III secretion system tip protein (SipD): A molecular docking and MD simulation study. *Informatics in Medicine Unlocked* **9**, 166-172 (2017).
49. Wang, D.*, et al.* Identification of bacterial target proteins for the salicylidene acylhydrazide class of virulence-blocking compounds. *J Biol Chem* **286**, 29922-29931 (2011).
50. Engstrom, P.*, et al.* Mutations in hemG mediate resistance to salicylidene acylhydrazides, demonstrating a novel link between protoporphyrinogen oxidase (HemG) and Chlamydia trachomatis infectivity. *J Bacteriol* **195**, 4221-4230 (2013).
51. Layton, A.N.*, et al.* Salicylidene acylhydrazide-mediated inhibition of type III secretion system-1 in Salmonella enterica serovar Typhimurium is associated with iron restriction and can be reversed by free iron. *FEMS Microbiol Lett* **302**, 114-122 (2010).
52. Slepenkin, A.*, et al.* Reversal of the antichlamydial activity of putative type III secretion inhibitors by iron. *Infection and immunity* **75**, 3478-3489 (2007).
53. Martinez-Argudo, I.*, et al.* Isolation of Salmonella mutants resistant to the inhibitory effect of Salicylidene acylhydrazides on flagella-mediated motility. *PloS one* **8**, e52179-e52179 (2013).
54. Zambelloni, R.*, et al.* Novel compounds targeting the enterohemorrhagic Escherichia coli type three secretion system reveal insights into mechanisms of secretion inhibition. *Mol Microbiol* **105**, 606-619 (2017).
55. Morgan, J.M.*, et al.* An Experimental Pipeline for Initial Characterization of Bacterial Type III Secretion System Inhibitor Mode of Action Using Enteropathogenic Yersinia. *Front Cell*

Infect Microbiol **8**, 404 (2018).
56. Kauppi, A.M., *et al.* Inhibitors of type III secretion in Yersinia: design, synthesis and multivariate QSAR of 2-arylsulfonyl-amino-benzanilides. *Bioorganic & medicinal chemistry* **15**, 6994-7011 (2007).
57. Duncan, M.C., *et al.* An NF-kappaB-based high-throughput screen identifies piericidins as inhibitors of the Yersinia pseudotuberculosis type III secretion system. *Antimicrob Agents Chemother* **58**, 1118-1126 (2014).
58. Niemz, A., *et al.* Point-of-care nucleic acid testing for infectious diseases. *Trends Biotechnol* **29**, 240-250 (2011).

EXAM QUESTIONS

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

We challenge you!

Question 1

Antibodies against IL-17, the most relevant product of Th17-cells, are used to treat patients with psoriasis. While undergoing treatment with these antibodies, patients have an increased risk of developing …

A. Hay fever
B. Fungal infections
C. Viral infections

(Topic from Q4-2 MGZ Immune system, 2019)

Question 2

A physician suspects that their patient is infected with the Epstein-Barr virus (EBV). EBV serology was ordered to strengthen this hypothesis, and the following result were obtained: EBV-IgM positive, EBV-IgG negative. What conclusions can be made upon these laboratory results? The patient has a ...

A. Primary immune deficiency
B. Primary EBV-infection
C. Reactivation of a previous EBV-infection

(Topic from Q4-1 MGZ Immune system, 2017)

The answers to these questions can be found on page 30 in this journal.

MYTH OR SCIENCE?
THE POWER OF PHEROMONES

Femke van Hout[1]

[1]Master's student Molecular Mechanisms of Disease, Radboud university medical center, Nijmegen, the Netherlands

Critical Appraisal

"Make your crush go crazy over you with only a couple of sprays of this pheromone cologne", "appeal to his desires" or "keep grabbing attention from women" are some of the promises seen in pheromone perfume adverts. An even bigger trust in the power of pheromones is seen at so-called "Pheromone Parties". The participating singles wear the same T-shirt the three nights before, bring it in a zip bag to the party and are given a number [1]. After a night of smelling the numbered t-shirts, matching singles can then decide to meet up. However, will we really meet our significant other through the smell of these specific molecules? And can pheromones really boost our sexual attraction? This article aims to explore the science behind such claims on pheromones in our mating behaviour and choice.

Pheromones (pherein: to transfer, hormon: that which excites) are often associated with sexual attraction and partner choice. However, the scientific origin of pheromones is not that sexy at all. In the 1930s, insect researchers were the first to distinguish chemical signals into endohormones and ectohormones [2]. Endohormones would be what we still call 'hormones' these days, such as the stress hormone cortisol and the sex hormone testosterone. These signalling molecules are secreted into the blood by specific glands [3]. The ectohormones entailed those 'hormones' that were secreted from the body of insects. A wide variety of functions has been ascribed to such ectohormones of insects, including the trailing of routes to their nest and alarming for predators [4, 5]. Only in 1959 were ectohormones renamed to pheromones and associated with sexual attraction by studies focusing on the silk moth [6]. The female silk moth produces Bombykol, a single molecule attracting the attention of every male moth around (Figure 1A) [7]. Darwin had already speculated on such a function of strong-smelling mammals before, stating that "the most odoriferous males are the most successful in winning the females" [8]. Yet, in contrast to the insect pheromones, only a few examples of pheromones have been described in mammals. This can in large part be attributed to the complex social behaviour of mammals, that is heavily intertwined with both context and past experiences [9]. Among the few compounds that have been linked to reproductive behaviour of mammals is androstenone. Male pigs produce and secrete androstenone, which then induces lordosis (mating readiness) in female pigs (Figure 1B) [10]. Androstenone can even be bought commercially by pig farmers to increase reproductive success. The finding of androstenone-like compounds under the armpits of humans led to wild speculations on human pheromones and even a market of pheromone perfumes [11]. But is there really a scientific basis to these perfumes claiming to increase our sexual attraction?

The definition of pheromones

To evaluate the role of pheromones in the mating behaviour and choice of humans, we first have to define what a pheromone is. The original definition of pheromones from 1959 states that pheromones are "substances secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process" [6]. This definition was, however, tailored to insect biology, and redefinitions to apply the concept of pheromones to mammals have never reached consensus [12, 13]. Nevertheless, most of the proposed definitions include that pheromones (a) are comprised of one or only a few compounds, (b) are species-specific, (c) have well-defined behavioural or endocrine effects, and (d) are little influenced by learning [12]. Indeed, the androstenone example of pigs concerns only one compound and has a stereotyped response in the female pigs, independent of their past experiences [10]. However, the species-specific criterium would no longer hold if androstenone indeed has similar effects on human females.

The putative human pheromones

The difficulties with the definition of pheromones did not stop the commercialisation of androstenone-like compounds in human products. This commercialisation was further supported by over forty papers that claim physiological and psychological effects of these 'putative pheromones' [14]. On the other hand, a large body of scientific critique has been published as well. Opponents would ask remarkable but critical questions such as "are women attracted to the odours of male pigs?" and "are birth rates higher in countries with pig farms?" [12]. Criticisers point out the use of non-physiological concentrations, small sample sizes, statistical errors, positive publication bias, and experimenter phenomena (where subjects are primed to expect the desired effect) in the forty studies [12, 15]. Moreover, not everybody is able to smell androstenone and related steroids, and the persons who do often find them unpleasant [16]. All in all, the opponents state that these putative pheromones have never been shown to be biologically relevant in humans and, therefore, should not be called pheromones.

The quest continues

But if these androstenone-like compounds are not the human pheromones, could there be other human pheromones? The slightly disappointing answer is that we do not know yet; we are not even sure whether human pheromones exist. Nevertheless, many scientists anticipate that there are human pheromones yet to be identified [15]. Like many other mammals, we also undergo changes in smell-producing secretions as we go through puberty that could function in sexual behaviour, and we have a good sense of smell [17]. Our difficulty with abstract thinking about smells seems to be more a cultural than a biological deficiency, related to an underappreciation of smell in the Western world [18]. However, unlike statements of pheromone perfume adverts, we no longer have a functional 'second nose', the vomeronasal organ, that many other animals use to

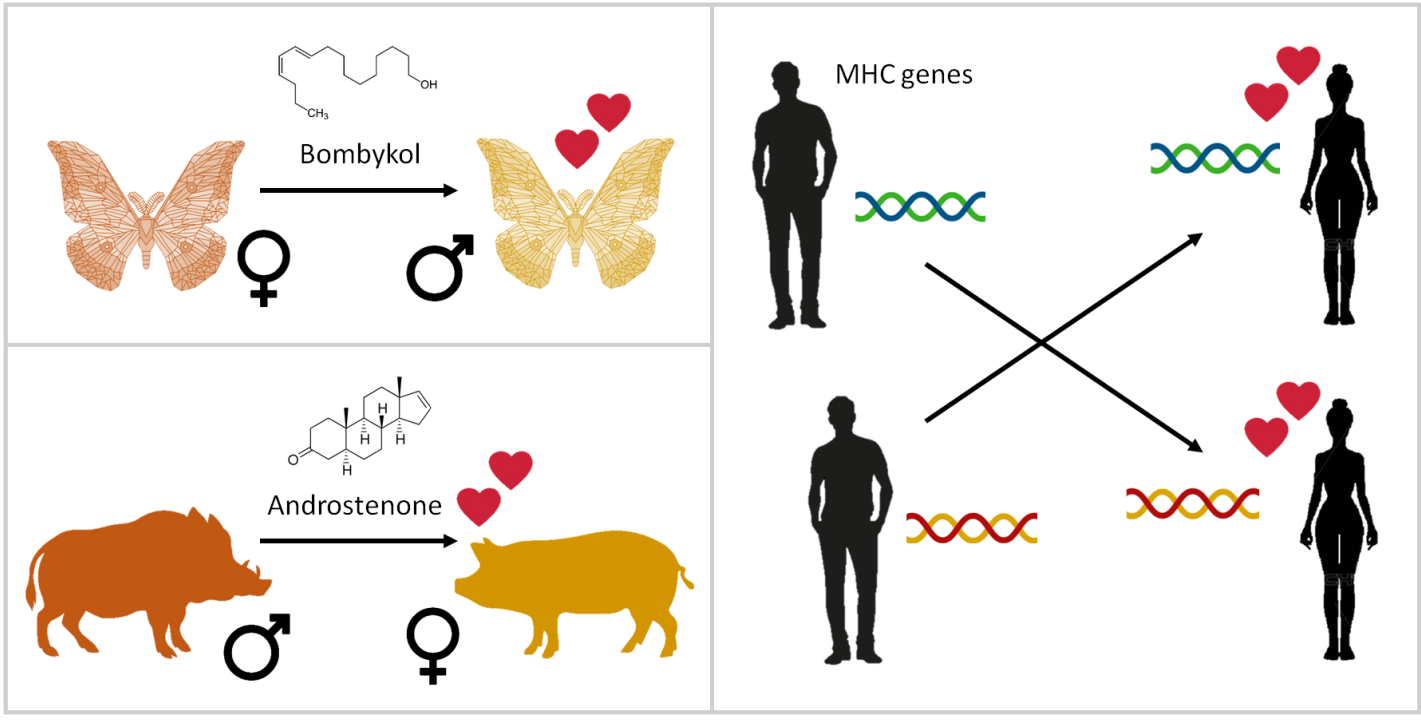


Figure 3: The role of smell in sexual behaviour and mate selection across the animal kingdom
In silk moths, females produce the molecule Bombykol to attract male moths (A). Male pigs produce the molecule androstenone, which induces mating readiness of female pigs (B). Humans may use the sense of smell in a more complicated way as women prefer the smell of men with MHC genes dissimilar to their own (C).

detect pheromones [19-21]. This vomeronasal organ is actually found within the nose and consists of sensory neurons that detect liquid organic compounds. However, in humans, this organ is believed to be vestigial, like our tailbone, and non-functional. If we would be able to (unconsciously) smell pheromones, we would have to detect them with our main olfactory system. Nevertheless, based on such reports in rabbits and sheep, we can conclude that, biologically speaking, there is no reason to think that humans do not use pheromone or pheromone-like molecules as social information [15]. However, the identification of human pheromones has proven to be a challenge, since up to this date, not one compound has been isolated that meets all the criteria of the pheromone definition. The difficulties in finding human pheromones lay in this strict definition of pheromones, the required study designs that are hard to translate from animal to human research, and the possibility that we may have lost our responses to pheromones [12, 15]. It is almost impossible to perform an adequately controlled trial, as human judgements of smell heavily depend on context and years of learning [9]. The continued quest for pheromones may, therefore, require adaptation of the pheromone concept to also address the individual differences in body odour production and perception.

Smelling T-shirts

Even though no human pheromones have been identified yet, we have a strong belief in the power of smell. Then where does this strong belief come from? Apart from pheromones, all kinds of non-pheromone smells affect our physiology and mood. Famous examples include the odours of rose oil to lower blood pressure, and lemon oil to enhance positive mood [22, 23]. Also, our own body odours can in fact influence others [24]. What we smell like is mainly determined by our diet, age, gender, and genetic make-up [9]. In addition, how we perceive the smell of others seems to vary between individuals [9]. A famous example of non-pheromone individual odours brings us to the 'sweaty T-shirt' experiments from 1995 [25]. Men were asked to sleep in the same T-shirt for two nights in a row and not to use odour-producing products. Women were asked to rate the scent of these T-shirts for pleasantness. Remarkably enough, women preferred the T-shirts of men that had variations in the *major histocompatibility complex* (MHC) genes different from their own (Figure 1C). These MHC genes, in humans also known as *human leukocyte antigen* genes, are important in the presentation of antigens to immune cells, and thereby play an essential role in the defence against pathogens [26]. The small but specific variations in these MHC genes determine which antigens will be presented, and thereby which pathogens can be attacked. The large individual differences in MHC variants can trouble organ transplantation with transplantation rejections, but also ensure a wide recognition of pathogens on a population level. On an individual level, MHC-dependent mate selection would allow women to choose an immunological complementary partner, such that their children would have a larger MHC diversity to recognise more pathogens. This would mean that humans use smell to select mates with favourable genes!

An immunological complementary mate

To better grasp this MHC-dependent odour selection, it is important to gain insight into the underlying biological basis. It seems that indeed, humans produce MHC-dependent odours [27, 28]. Although it remains unclear how exactly MHC molecules lead to specific odour profiles, trained rats can discriminate between the urine of people with different MHC genes [29]. Moreover, humans also show MHC-dependent odour perception [30]. Even the preferences in choosing our own perfumes seem to correlate with our MHC genotype, leading to speculations that perfume use has evolved as a means to advertise our own MHC type for potential mates [31]. Yet, the 'sweaty T-shirt' experiment from 1995 has been repeated over and over with only variable success [32]. Proponents of the theory state that the importance of MHC-dependent mate selection differs between study populations. Under certain contexts and in different geographic regions, MHC dissimilarity preferences may be stronger [27]. Opponents state that MHC-dependent mate selection

may have existed, as it is scientifically widely sustained in for example mice and rats, but that it is greatly overruled by cultural and social factors.

Conclusion

All in all, it seems that the term pheromone is confusing and often misused and that not one human pheromone has been conclusively identified yet [15]. But poor scientific validity of the pheromone perfumes does not mean that smell is not important in mating. Smells are actually very important in recognition and bonding, and possibly even in the finding of an immunologically complementary partner [32]. However, these smells are our individual smells and are not perceived the same by every person of the human species, and should therefore not be confused with or misnamed as pheromone molecules [15]. While the scientific quest for human pheromones continues, it would not harm you to take a good sniff of your potential future mates. In the end, a "Complementary Immune Gene Party" may not sound as sexy as a "Pheromone Party", but it might be a nice party after all.

Acknowledgements

RAMS would like to thank Jasper de Groot, PhD, from the Behavioural Science Institute, Radboud University Nijmegen, for proofreading this article and providing feedback.

References

1. Subramanian, C. Can your smelly shirt land you a better first date? *TIME* (2012).
2. Benthe, A. Vernachlässigte hormone. *Naturwissenschaften* **20**, 177-181 (1932).
3. Kronenberg, H. M., *et al*. *Chapter 1 – Principles of Endocrinology*. In Williams Textbook of Endocrinology (Elsevier, Philadelphia, 2016).
4. Wilson, E. O. Chemical communication among workers of the fire ant Solenopsis saevissima. *Animal behaviour* **10**, 134-147 (1962).
5. Verheggen, F. J., *et al*. Alarm pheromones-chemical signaling in response to danger. *Vitamins and hormones* **83**, 215-239 (2010).
6. Karlson, P. & Luscher, M. Pheromones: a new term for a class of biologically active substances. *Nature* **183**, 55-56 (1959).
7. Butenandt, A., *et al*. Über den sexuallockstoff des seidenspinners, I. Der biologische test und die isolierung des reinen sexuallockstoffes Bombykol. *Biological Chemistry* **324**, 71 (1961).
8. Darwin, C. *The descent of man and selection in relation to sex,* (D. Appleton, 1896).
9. De Groot, J. H. B., *et al*. On the communicative function of body odors: A theoretical integration and review. *Perspectives on Psychological Science* **12**, 306-324 (2017).
10. Melrose, D.R., *et al*. Androgen steroids associated with boar odour as an aid to the detection of oestrus in pig artifical inseminatino. *British Veterinary Journal* **127**, 497-502 (1971).
11. Nixon, A., *et al*. Simultaneous quantification of five odorous steroids (16-androstenes) in the axillary hair of men. *Journal of steroid biochemistry* **29**, 505-510 (1988).
12. Doty, R. *Human pheromones*. In Neurobiology of Chemical Communicatino (CRC Press/Taylor & Francis, Boca Raton, 2014).
13. Katz, R. A., *et al*. In Defense of the term "Pheromone". *Journal of Chemical Ecology* **5**, 299-305 (1979).
14. Havlicek, J., *et al*. Current issues in the study of androstenes in human chemosignaling. *Vitamins and hormones* **83**, 47-81 (2010).
15. Wyatt, T. D. The search for human pheromones: the lost decade and the necessity of returning to first principles. *Proceedings of the Royal Society B: Biological Sciences* **282**, 20142994 (2015).
16. Gower, D. B., *et al*. Axillary 5 alpha-androst-16-en-3-one in men and women: relationships with olfactory acuity to odorous 16-androstenes. *Experientia* **41**, 1134-1136 (1985).
17. Wyatt, T. D. *Pheromones and animal behavior: chemical signals and signatures* (Cambridge University Press, Cambridge, 2014).
18. Majid, A. & Burenhult, N. Odors are expressible in language, as long as you speak the right language. *Cognition* **130**, 266-270 (2014).
19. Witt, M. & Hummel, T. Vomeronasal versus olfactory epithelium: Is there a cellular basis for human vomeronasal perception? *International Review of Cytology* **248**, 209-259 (2006).
20. Smith, T. D., *et al*. The shrinking anthropoid nose, the human vomeronasal organ, and the language of anatomical reduction. *The Anatomical Record* **297**, 2196-2204 (2014).
21. Baxi, K.N., *et al*. Is the vomeronasal system really specialized for detecting pheromones? *Trends in neurosciences* **29**, 1-7 (2006).
22. Haze, S., *et al*. Effects of fragrance inhalation on sympathetic activity in normal adults. *Japanese journal of pharmacology* **90**, 247-253 (2002).
23. Kiecolt-Glaser, J.K., *et al*. Olfactory influences on mood and autonomic, endocrine, and immune function. *Psychoneuroendocrinology* **33**, 328-339 (2008).
24. Lundström, J.N., *et al*. Functional neuronal processing of body odors differs from that of similar common odors. *Cerebral Cortex* **18**, 1466-1474 (2007).
25. Wedekind, C., *et al*. MHC-dependent mate preferences in human. *Proceedings. Biological sciences* **260**, 245-249 (1995).
26. Sommer, S. The importance of immune gene variability (MHC) in evolutionary ecology and conservation. *Front Zool* **2**, 16-16 (2005).
27. Penn, D.J. & Potts, W.K. The evolution of mating preferences and major histocompatibility complex genes. *The American Naturalist* **153**, 145-164 (1999).
28. Boehm, T. & Zufall, F. MHC peptides and the sensory evaluation of genotype. *Trends in neurosciences* **29**, 100-107 (2006).
29. Eggert, F., *et al*. The major histocompatibility complex and the chemosensory signalling of individuality in humans. *Genetica* **104**, 265-273 (1998).
30. Janeš, D., *et al*. Influence of MHC on odour perception of 43 chemicals and body odour. *Central European Journal of Biology* **5**, 324-330 (2010).
31. Milinski, M. & Wedekind, C. Evidence for MHC-correlated perfume preferences in humans. *Behavioral Ecology* **12**, 140-149 (2001).
32. Havlicek, J. & Roberts, S.C. MHC-correlated mate choice in humans: A review. *Psychoneuroendocrinology* **34**, 497-512 (2009).

MULTIPLE SCLEROSIS GOES VIRAL: THE IMPACT OF VIRAL INFECTIONS ON THE DEVELOPMENT OF MULTIPLE SCLEROSIS

Natalie Ludwig[1]

[1] Master's student Molecular Mechanisms of Disease, Radboud university medical center, Nijmegen, The Netherlands

Abstract

Review

Multiple Sclerosis (MS) is an autoimmune disease of the central nervous system that has fascinated and bewildered scientists for decades. Patients suffering from MS have an impaired immune tolerance resulting in the emergence of autoreactive immune cells, which infiltrate the brain and spinal cord and attack the myelin sheath of nerves. While the prevalence pattern indicates a genetic component to the development of MS, environmental influences have been identified as crucial players as well, including certain viral infections. Proposed viruses include Epstein-Barr virus, human cytomegalovirus, and endogenous retroviruses, amidst others. The interplay of viral infections and MS development is complicated, and many questions remain unanswered, most notably how viruses exactly increase MS susceptibility. Elucidating these mechanisms could provide valuable insights into immune regulation processes and might be transferable to other immune diseases. Furthermore, a better understanding of the disease aetiology could substantially improve treatments, which are unsatisfying as of now. Thus, research efforts in this exciting field could open up new possibilities for treatments and, hence, in the long term, significantly increase the quality of life of MS patients. This article aims to briefly outline the role of the three aforementioned viruses in MS development and highlight the therapeutical potential of better understanding the connection between viral infections and MS risk.

KEYWORDS: Autoimmune disease, Environmental risk factor, Epstein-Barr virus, Human cytomegalovirus, Endogenous retroviruses

For a long time, autoimmune processes were reckoned to be impossible and long after their introduction in the mid 20th century by Paul Ehrlich, the concept remained highly controversial [1, 2]. Nonetheless, with time, more and more diseases were shown to have autoimmune processes underlying, making autoimmune conditions an important and relatively common family of diseases [3]. One of these diseases is Multiple Sclerosis (MS), an autoimmune condition manifesting in the central nervous system (CNS) with an intriguing prevalence pattern [4]. While MS is rather common in high-income countries (140 and 108 cases per 100,000 individuals for North America and Europe, respectively), it occurs to a much lesser extent in regions like East-Asia and Sub-Saharan Africa with approximately two cases per 100 000 individuals [4]. Several viral infections have been proposed to act as environmental factors, including Epstein-Barr virus (EBV), human cytomegalovirus (CMV), and members of the human endogenous retrovirus family W (HERV-W) [5-7]. However, no concrete conclusion to the debate has been obtained yet. Thus, this review aims to give a broad overview of the disease and the viruses in question, before outlining the current state of knowledge about the impact of viral infections in MS development and highlighting the translation potential of these findings.

Clinical description of the disease

MS is a chronic disease, characterised by inflammatory processes and demyelination events within the CNS, which describes the destruction of the myelin layer that normally isolates nerves from its surroundings [5]. Additionally, varying degrees of damage to neurons and their axons have been described in MS [5]. Symptoms of the disease vary depending on the location of the lesions in the brain and can include decreased control of movement and bladder function as well as reduced cognitive abilities [5]. Two different types of MS can be distinguished when it comes to disease progression [8]. One form is characterised by the appearance of timely restricted symptoms, so-called "relapses", which then vanishes again, giving this form of MS the name "relapsing-remitting MS" [8]. Over time, this form can transform into a secondary progressive state, which is characterised by the continuous worsening of symptoms [8]. However, in 10% of cases, the patient's health status slowly deteriorates from the inception, which is titled primary progressive MS [8].

Life expectancy seems to be similar between the general population and MS patients under 40 (hazard ratio (HR) referring to the likelihood that an individual meets the event, i.e. dies, of 0.63 (95% confidence interval (CI) 0.23–1.70)) [9]. The HR is slightly increased in patients aged 40-59 compared to individuals without MS (HR of 1.68 (95% CI 1.05–2.69)) [9]. Strikingly, the oldest patients (60 years of age and over) showed an HR of 11.37 (95% CI 8.12–15.93), compared to the overall population [9]. These statistics lead us to conclude a directly proportionate rate of mortality with age. Also, the overall HR was increased in the MS population, compared to individuals without MS (HR 3.51, 95% CI 2.63–4.69), which is backed up by previous studies [9-12]. While primary progressive MS was significantly associated with a decrease in life span (risk ratio 1.99; 95% CI 1.52–2.59), the mean age of patients at death was similar between primary progressive MS and relapsing-remitting MS (*p*-value 0.155) [10]. As of now, there is no curative treatment available for MS as the current approaches only slow down the disease progression but do not achieve to halt it completely [8].

Pathogenesis of MS

The pathogenesis of MS is complicated and not fully elucidated yet. As is the case for any autoimmune disease, it is hypothesised that a defective immune tolerance causes MS [13]. Under normal conditions, autoreactive

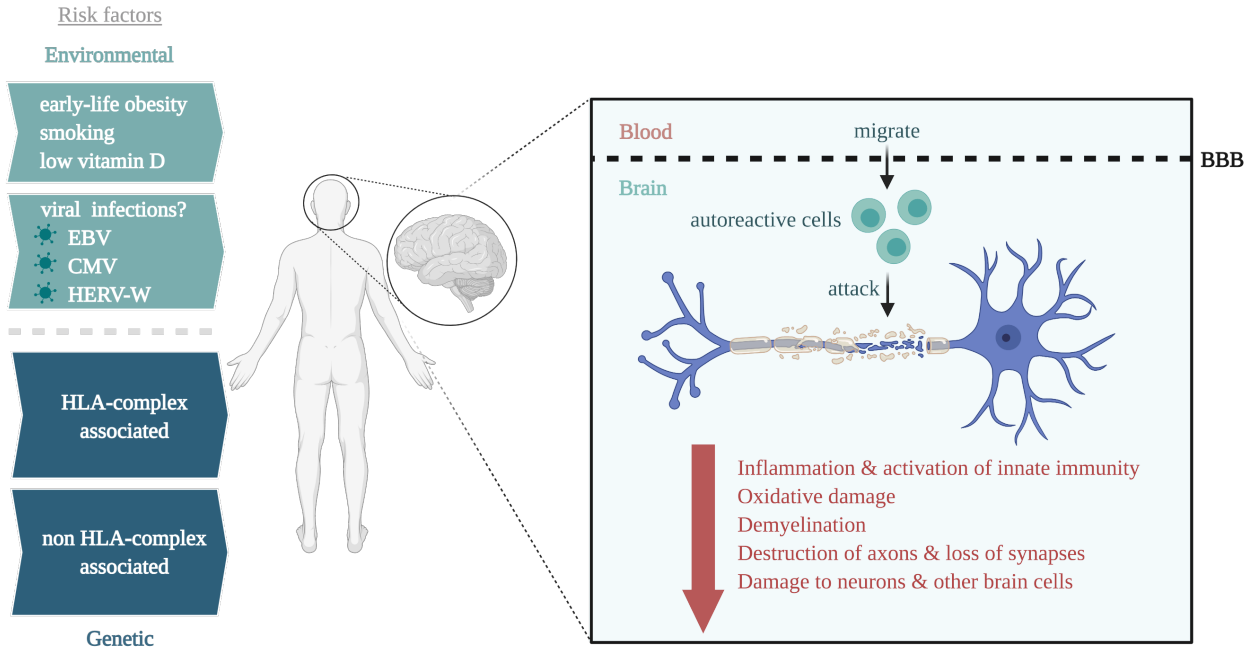


Figure 1: Proposed pathogenesis of multiple sclerosis

MS arises due to a complex interplay of genetic and environmental risk factors. Genetic risk factors include human leukocyte antigen (HLA) complex-associated and non-HLA complex-associated alleles. Regarding environmental risk factors, several are thought to impact MS risk, including early-life obesity, smoking, and low serum vitamin D levels. Furthermore, also certain viral infections might be facilitating MS development, including Epstein-Barr virus (EBV), human cytomegalovirus (CMV) and, members of the human endogenous retrovirus family W (HERV-W). These risk factors promote the emergence of autoreactive cells that migrate into the central nervous system (CNS) via the blood-brain barrier (BBB). There they attack the myelin covering of neurons. This process results in demyelination, destructions of axons, loss of synapses, and damage to neurons and other brain cells, as well as inflammation and activation of the innate immune system and oxidative damage. Created with BioRender.com.

immune cells of the adaptive immune system are inactivated or removed by apoptosis. However, in MS, they are thought to persist and become activated [13]. These cells then enter the CNS and damage the myelin isolation of neurons, resulting in demyelination, as well as the loss of synapses, axonal injury, and damage to neurons and other brain cells [13]. These localised spots of tissue damage, called "demyelinated plaques", show up as lesions on MRI scans and are associated with the further breakdown of the blood-brain barrier [13]. Due to the increase in barrier permeability, there is enhanced leukocyte infiltration into the CNS [13]. Within lesions and the surrounding tissue, several immune cell types are present, including B- and T-lymphocytes, monocyte-derived macrophages, natural killer cells, neutrophils, and microglia [14-16]. The damage is only insufficiently repaired and gets exacerbated by processes involving the activation of innate immunity in the CNS and oxidative damage, amongst others [13]. Combined with the exhaustion of the compensatory mechanisms of the CNS over time, these processes then lead to further decline of neurological functions [13].

In earlier times, MS was thought to be primarily a T-cell-mediated disease and there is ample data to substantiate this [17, 18]. However, several clinical trials were able to show that the use of B-cell depleting antibodies decreased disease activity, also indicating a role for B-cells in disease pathogenesis [19, 20]. The role of B-cells seems primarily to be centred around antigen presentation and the production of proinflammatory cyto- and chemokines as opposed to antibody production. This conclusion is based on studies showing a fast improvement of symptoms upon B-cell depleting therapy and others which determined the absence of autoantibodies [21].

Risk factors

So far, no single genetic determinant of MS has been reported [5]. A comparison of large population-based twin studies support a genetic contribution to MS risk; however, the extent is still subject to debate [22]. Instead of a sole genetic source, MS rather seems to arise due to a complex interplay between genetic risk factors and environmental factors [8]. Genetic risk factors are mainly found in loci associated with the human leukocyte antigen complex and thereby impact the presentation of antigens to T-lymphocytes [8]. Nevertheless, other gene variants have been implicated as well [8].

Currently, it is theorised that MS occurs in individuals who are susceptible to the disease due to their genetic background and additionally experience a combination of environmental factors [5]. The combination of environmental circumstances needed to initiate MS in a patient seems to be highly variable, differing both in the composition of factors as well as their individual contribution to MS progression [21]. The proposed environmental factors increasing MS risk are numerous and include smoking, obesity in early life, and low vitamin D levels [8]. Notably, also viral infections are discussed in their implication in augmenting MS risk, and some of the potential viral players are discussed below in an attempt to give an overview of the proposed interaction models.

Viral infections as environmental risk factors

Viral infections as initiators of autoimmune diseases have been extensively reviewed, with the conclusion that some viruses can definitely give rise to autoimmunity [23, 24]. However, for MS, the situation remains less clear [8]. There are several viruses discussed in connection to MS development, with some viruses remaining more controversial than others [8]. This review will focus on three examples, namely EBV, human CMV, and HERV-W (Figure 1) [5-7].

Of note, this is an active, dynamic field of research and therefore, new theories about how viral infections act in MS pathogenesis arise and are overturned almost on a daily basis [25]. The most substantiated hypotheses are outlined below, although the list is by no means exhaustive [25]. Firstly, there is molecular mimicry, which describes the induction of an immune response against a viral antigen, that is similar to a self-antigen, thereby potentially promoting an autoimmune response [25]. This can also result in epitope spreading, a process where the body starts fighting viral antigens, but due to the damage following the immune response, also self-antigens are released [25]. The immune system then falsely recognises them as viral antigens and induces an immune response, resulting in autoimmunity [25]. Another possible theory is titled "bystander activation" and describes a situation where viral products activate formerly inactive dendritic cells presenting self-antigens [25]. These then stimulate the emergence of autoreactive T-cells, thereby promoting autoimmunity [25]. However, in recent years, the bystander activation theory has been increasingly regarded as unlikely to be the major mechanism [25]. Lastly, it might be the case that two viral infections are needed to induce MS development, one acting as a priming factor and the other virus finally inducing MS years later [25]. This, however, has only so far been described in animal models of MS [25].

Research in this field is difficult, and often, the results are inconclusive [26]. One of the biggest hurdles is the small cohorts of eligible participants within a study [26]. This reduces the power of an analysis to pick up a certain effect and can potentially result in a lack of significance [26]. Furthermore, often important information on health aspects of the individual are not available for all participants, which impedes the analysis [26]. Finally, it needs to be acknowledged that correlation does not necessarily entail causation [6]. Thus, epidemiological evidence can never establish a causal link between two aspects and needs to be backed up with experimental data in MS models [6].

Epstein-Barr virus

EBV is a member of the family of herpesviruses and ubiquitously found in humans [5]. Following an EBV infection, EBV-specific antibodies can be found in individuals, who are then described as EBV seropositive [5]. Symptoms of EBV-induced disease differ depending on the age of the infected person [5]. While EBV infections in prepubertal individuals are asymptomatic, adolescents and adults can develop infectious mononucleosis (IM) [5]. IM is the clinical manifestation of acute EBV infections and characterised by symptoms like fever, inflammation of the throat, and enlargement of the lymph nodes [5, 27].

EBV has been associated with the occurrence of MS following a number of epidemiological studies that investigated the correlation between EBV seropositivity or a history of IM, and MS in individuals. Firstly, in a serological study from 2011, all included MS patients were either initially seropositive for EBV or turned seropositive before the onset of MS [28]. Secondly, a meta-analysis of 18 studies highlighted that the risk of developing MS is two- to three-fold higher for individuals with a recorded history of IM than the risk for individuals that never experienced IM [29]. Conversely, seronegativity seems to be negatively associated with MS [30]. A meta-analysis calculated the MS risk of a member of the EBV seronegative cohort at 0.06, which is decidedly lower than average (95% CI 0.03-0.13) [30]. Lastly, this correlation is backed up by serological data, which correlated the risk of MS with EBV-specific antibodies [28]. In the largest study, conducted by Munger *et al.* in 2011, a striking correlation was found between the levels of immunoglobulin G antibodies targeting the Epstein-Barr nuclear antigen (EBNA) complex and MS risk (p-value $2.1E^{-13}$) [28]. Furthermore, similar trends also appeared concerning other EBV-specific antibodies and the risk of developing MS (p-values $5.7E^{-9}$ for EBNA1, $9E^{-4}$ for EBNA2, and $5.7E^{-7}$ for the viral capsid antigen) [28].

The exact mechanism of how EBV infection influences the pathogenesis of MS is not fully elucidated yet; however, numerous theories are currently being investigated. Interestingly, cross-reactivity of EBNA1 with human heterogeneous nuclear ribonucleoprotein L, a known autoantigen in MS, has been described [31]. This strengthens the idea of molecular mimicry. Furthermore, also EBV-specific mechanisms are debated, such as the leakage of EBV-infected B-cells into the CNS, where they induce a pro-inflammatory environment [5, 25].

Notably, rather than exposure to the virus itself (i.e. EBV seropositivity), the clinical manifestation of the EBV-infection in the form of IM seems to determine the risk of developing MS [30]. This phenomenon can be explained by the hygiene hypothesis [30]. It states that early exposure to infections can aid the formation of immunoregulatory mechanisms, which then convey protection against pathogenicity of autoreactive cells [32, 33]. The hygiene hypothesis is substantiated by evidence derived from other prevalent pathogens in the context of MS, like *Helicobacter pylori* [34]. Due to MS being a multifactorial disease, it is, up to this day, difficult to determine causality between EBV and MS, despite the extensive evidence [8]. More research will be necessary to elucidate mechanisms by which EBV could induce MS.

Human cytomegalovirus

Human CMV is a member of the family of herpesviruses (just like EBV) and ubiquitously present in adults [6]. The role of CMV in MS development is highly controversial and both evidence for a protective as well as for a harmful role has been acquired [6]. In a recent transethnic case-control study investigating CMV seropositivity in correlation with MS risk, CMV was shown to have a protective effect [35]. However, this effect was only found in Hispanic individuals, as opposed to black or white study participants [36]. This raises the possibility that the protective effect is actually due to a yet to be determined confounder, instead of CMV [36]. Additionally, a meta-analysis of previously published studies performed by Pakpoor *et al.* in 2013 did not find conclusive evidence in favour of an association between CMV infections and decreased MS risk [36].

Nevertheless, one study described a reduced likelihood for pediatric-onset MS, which is characterised by an emergence of the disease during childhood, in the case of CMV seropositivity [37]. This was later broadened to all types of MS when, in 2013, a meta-analysis claimed CMV infection to be associated with a lower MS risk in general [26]. However, both studies are retrospective, which leaves room for doubts [26]. For instance, during the long periods between initial serological tests and the following classification of MS progression, initially seronegative patients can become infected with the virus [26]. This could potentially skew the results [26]. Additionally, the statistics of retrospective meta-analysis can be misleading [26]. In the meta-analysis performed by Sundquist *et al.* in 2013, for example, eleven studies were included, of which only two initially showed a significant association between CMV seropositivity and MS development [26]. Nevertheless, the overall meta-analysis also concluded that CMV seropositivity and MS risk are significantly associated [26].

To conclude, the effect of CMV infection on the risk of developing MS remains highly debated, and no conclusive evidence has been brought forward to explain the complex interplay. In an attempt to explain the outlined inconsistencies, the possibility has been raised that CMV may have opposite effects on MS development and the course of the disease and may have stronger detrimental effects at later stages [33].

Endogenous retroviruses

Endogenous retroviruses (ERVs) describe repetitive genomic sequences which are thought to derive from retroviruses infecting germline cells and integrating into their host cell genome [38]. In humans, they are no longer active as infectious viruses, although both viral RNA and proteins are expressed. Furthermore, interactions between the regulatory elements within the viral genome and cellular transcription factors have been identified [38]. This raises the possibility that the consistent presence of viral products impacts cellular processes, including (auto-)immunity [38].

The association of MS and human ERVs (HERVs) is highly controversial, albeit several systematic reviews and meta-analysis recently illustrated that, compared to healthy individuals, MS patients over-express RNA from a specific HERV family, namely the HERV-W family [7]. Other ERV families are also discussed in the context of MS; however, the gathered evidence is less strong [7]. HERV-W is constitutively expressed in the CNS [39]. The proposed mechanism of how HERV-W members facilitate the development of MS centres around the expressed envelope proteins of these endogenous retroviruses [40]. These proteins can activate Toll-like receptor 4 *in vitro*, which promotes the secretion of several proinflammatory cytokines [40]. HERV-W proteins further were shown to lead to the development of neuroinflammation, as well as myelin and oligodendrocyte damage *in vivo* [40]. In fact, the envelope protein of HERV-W is considered as a superantigen, which, after administration to mice, induces systemic inflammation [40]. Of note, systemic inflammation has also been observed in MS in the form of peripheral T-cell activation [40]. Expression of the HERV-W envelope protein has been described in microglial cells and macrophages in MS brains close to MS lesions and might there drive neuroinflammation [40]. However, proteins of other HERV families were shown to act immunosuppressive by inducing immunomodulatory macrophages, which makes the picture more facetted [41]. Thus, a consensus of the impact of HERV products on MS risk is yet to be reached, and more research will be necessary to elucidate the exact effect HERV family members have on MS development.

Translational potential

While MS is, in most cases, not directly fatal, it does have a substantial impact on the quality of life and can reduce life expectancy [9, 42]. Furthermore, biomarkers to recognise at-risk individuals for MS are still lacking [25]. In addition, albeit there are treatments available, they do not achieve satisfying results for all types of MS progressions [8]. Therefore, there is still an unmet clinical need for better treatments to actually halt disease progression. A better understanding of the interaction between viral infections and MS could enable us to discover biomarkers to (i) identify individuals with a higher MS risk and (ii) predict disease progression in patients diagnosed with MS, while also aiding in the development of new therapeutic approaches [25]. As of 2017, several treatments primarily targeting viruses underwent trials of varying clinical phases in the context of MS [43]. Most notably, the antiviral compound acyclovir and its prodrug valacyclovir targeting herpesviruses took part in three phase III trials in the last decades [43]. Upon subgrouping the patients according to disease activity, treatment resulted in a 34% reduction in relapse rate in patients with high disease activity [44]. Another study indicated a decrease in baseline disease activity following anti-herpes virus therapy in comparison to a placebo-treated group due to a decreased number of active CNS lesions in MS patients undergoing treatment [45]. Thus, while the primary ends of the trials were not met, the results look promising nonetheless and give hope for the use of antiviral drugs in treating MS [43].

Conclusion

In conclusion, MS remains a medical challenge due to the elusive disease pathogenesis, lack of prognostic biomarkers and inadequate treatment options. The complex interplay between genetic and environmental risk factors further increases the difficulties in preventing and treating MS. Understanding how viral infections interact with the MS risk in genetically susceptible individuals will be pivotal in understanding the molecular mechanisms behind the disease and finding better treatment approaches. Furthermore, findings in this field might offer valuable insights for other autoimmune diseases as well. Antiviral treatments hold the potential to meet a current gap in medical care and improve the quality of life for MS patients.

Acknowledgements

RAMS would like to thank Arjan van Caam, PhD, from the department of Experimental Rheumatology of the Radboudumc, for providing feedback, as well as Aman Urazov, BSc and Rojina Duwal, BSc for their creative input.

References

1. Ehrlich, P. *The Collected Papers Of Paul Ehrlich. Vol II: Immunology And Cancer Research*, (Pergamon Press, London, 1957).
2. Margo, C.E. & Harman, L.E. Autoimmune disease: Conceptual history and contributions of ocular immunology. *Surv Ophthalmol* **61**, 680-688 (2016).
3. Rose, N.R. In the beginnings. *The Israel Medical Association Journal* **17**, 74-79 (2015).
4. Leray, E., *et al.* Epidemiology of multiple sclerosis. *Rev Neurol* **172**, 3-13 (2016).
5. Nourbakhsh, B. & Mowry, E. Multiple sclerosis risk factors and pathogenesis. *CONTINUUM (MINNEAP MINN)* **25**, 596-610 (2019).
6. Vanheusden, M., *et al.* Cytomegalovirus: a culprit or protector in multiple sclerosis? *Trends Mol Med* **21**, 16-23 (2015).
7. Morandi, E., *et al.* The association between human endogenous retroviruses and multiple sclerosis: A systematic review and meta-analysis. *PLoS One* **12**, e0172415 (2017).
8. Olsson, T., *et al.* Interactions between genetic, lifestyle and environmental risk factors for multiple sclerosis. *Nat Rev Neurol* **13**, 25-36 (2017).
9. Lalmohamed, A., *et al.* Causes of death in patients with multiple sclerosis and matched referent subjects: a population-based cohort study. *Eur J Neurol* **19**, 1007-1014 (2012).
10. Smestad, C., *et al.* Excess mortality and cause of death in a cohort of Norwegian multiple sclerosis patients. *Mult Scler* **15**, 1263-1270 (2009).
11. Sumelahti, M.L., *et al.* Causes of death among patients with multiple sclerosis. *Mult Scler* **16**, 1437-1442 (2010).
12. Bronnum-Hansen, H., *et al.* Trends in survival and cause of death in Danish patients with multiple sclerosis. *Brain* **127**, 844-850 (2004).
13. Mahad, D.H., *et al.* Pathological mechanisms in progressive multiple sclerosis. *The Lancet Neurology* **14**, 183-193 (2015).
14. De Bondt, M., *et al.* Neutrophils: Underestimated Players in the Pathogenesis of Multiple Sclerosis (MS). *Int J Mol Sci* **21**(2020).
15. Alvarez, J.I., *et al.* Focal disturbances in the blood-brain barrier are associated with formation of neuroinflammatory lesions. *Neurobiol Dis* **74**, 14-24 (2015).

16. Van Kaer, L.*, et al.* Innate, innate-like and adaptive lymphocytes in the pathogenesis of MS and EAE. *Cell Mol Immunol* **16**, 531-539 (2019).
17. Danikowski, K.M.*, et al.* Regulatory T cells in multiple sclerosis and myasthenia gravis. *J Neuroinflammation* **14**, 1-17 (2017).
18. Kaskow, B.J. & Baecher-Allan, C. Effector T Cells in Multiple Sclerosis. *Cold Spring Harb Perspect Med* **8**, 1-14 (2018).
19. Hauser, S.L.*, et al.* B-cell depletion with rituximab in relapsing-remitting multiple sclerosis. *N Engl J Med* **358**, 676–688
20. (2008).
21. Hauser, S.L.*, et al.* Ocrelizumab Versus Interferon Beta-1a in Relapsing Multiple Sclerosis. *N Engl J Med* **376**, 221-234 (2017).
22. Goodin, D.S. The causal cascade to multiple sclerosis: a model for MS pathogenesis. *PLoS One* **4**, e4565 (2009).
23. Hawkes, C. & Macgregor, A. Twin studies and the heritability of MS: a conclusion. *Multiple Sclerosis* **15**, 661–667 (2009).
24. Smatti, M.K.*, et al.* Viruses and Autoimmunity: A Review on the Potential Interaction and Molecular Mechanisms. *Viruses* **11**(2019).
25. Getts, D.R.*, et al.* Virus infection, antiviral immunity, and autoimmunity. *Immunol Rev* **255**, 197-209 (2013).
26. Mentis, A.A.*, et al.* Viruses and Multiple Sclerosis: From Mechanisms and Pathways to Translational Research Opportunities. *Mol Neurobiol* **54**, 3911-3923 (2017).
27. Sundqvist, E.*, et al.* Cytomegalovirus seropositivity is negatively associated with multiple sclerosis. *Mult Scler* **20**, 165-173 (2014).
28. Ebell, M.H. Epstein-Barr Virus Infectious Mononucleosis. *American Family Physician* **70**, 1279-1287 (2004).
29. Munger, K.L.*, et al.* Anti-Epstein-Barr virus antibodies as serological markers of multiple sclerosis: a prospective study among United States military personnel. *Mult Scler* **17**, 1185-1193 (2011).
30. Handel, A.E.*, et al.* An updated meta-analysis of risk of multiple sclerosis following infectious mononucleosis. *PLoS One* **5**, e12496 (2010).
31. Ascherio, A. & Munger, K.L. Environmental risk factors for multiple sclerosis. Part I: the role of infection. *Ann Neurol* **61**, 288-299 (2007).
32. Lindsey, J.W.*, et al.* Antibodies specific for Epstein-Barr virus nuclear antigen-1 cross-react with human heterogeneous nuclear ribonucleoprotein L. *Mol Immunol* **69**, 7-12 (2016).
33. Strachan, D.P. Hay fever, hygiene, and household size. **299**, 1259-1260 (1989).
34. Yazdanbakhsh, M.*, et al.* Allergy, parasites, and the hygiene hypothesis. *Science* **296**, 490-494 (2002).
35. Kira, J.I. & Isobe, N. Helicobacter pylori infection and demyelinating disease of the central nervous system. *J Neuroimmunol* **329**, 14-19 (2019).
36. Langer-Gould, A.*, et al.* Epstein-Barr virus, cytomegalovirus, and multiple sclerosis susceptibility. *Neurology* **89**, 1330-1337 (2017).
37. Pakpoor, J.*, et al.* Cytomegalovirus and multiple sclerosis risk. *J Neurol* **260**, 1658-1660 (2013).
38. Waubant, E.*, et al.* Common viruses associated with lower pediatric multiple sclerosis risk. *Neurology* **76**, 1989-1995 (2011).
39. Christensen, T. Human endogenous retroviruses in the aetiology of MS. *Acta Neurol Scand* **136** 18-21 (2017).
40. Perron, H.*, et al.* Human endogenous retrovirus (HERV)-W ENV and GAG proteins: physiological expression in human brain and pathophysiological modulation in multiple sclerosis lesions. *J Neurovirol* **11**, 23-33 (2005).
41. Morris, G.*, et al.* Do Human Endogenous Retroviruses Contribute to Multiple Sclerosis, and if So, How? *Mol Neurobiol* **56**, 2590-2605 (2019).
42. Bahrami, S.*, et al.* Immunomodulating peptides derived from different human endogenous retroviruses (HERVs) show dissimilar impact on pathogenesis of a multiple sclerosis animal disease model. *Clin Immunol* **191**, 37-43 (2018).
43. Rosiak, K. & Zagozdzon, P. Quality of life and social support in patients with multiple sclerosis. *Psychiatr Pol* **51**, 923-935 (2017).
44. Lycke, J. Trials of antivirals in the treatment of multiple sclerosis. *Acta Neurol Scand* **136**, 45-48 (2017).
45. Bech, E.*, et al.* A randomized, double-blind, placebocontrolled MRI study of anti–herpes virus therapy in MS. *NEUROLOGY* **58**, 31–36 (2002).
46. Lycke, J.*, et al.* Acyclovir treatment of RRMS - a randomized, placebo-controlled, double-blind study.pdf>. *J Neurol* **243**, 214-224 (1996).

BACTERIOPHAGES: A SUITABLE ALTERNATIVE FOR ANTIBIOTICS?

Quentin Marsman[1]

[1] BSc Medicine, Radboud university medical center, Nijmegen, the Netherlands

Insights

Imagine you feel a little ill and you decide to call the doctor. The doctor suspects a common bacterial infection of the urinary tract. After a failed treatment with a broad-spectrum antibiotic, additional examinations reveal a multi-resistant bacterium in your body, resistant to most of the available antibiotics. You are worried about this result, as some of your loved ones were also infected by such a bacterium in the past and have not recovered from the infection. You receive further treatment after which you barely recover.

Even though this short story sounds dystopian, it could become a reality in the future. Due to our current antibiotic usage, both in medicine and the cattle industry, antibiotic resistance is on the rise. This could lead to infections that pose a therapeutic challenge and might even prove to be lethal. Therefore, an alternative is clearly needed. A possible alternative that is currently being researched is the treatment with bacteriophages. Could these bacteria-killing viruses be the solution we need?

In its first report about antimicrobial resistance, the World Health Organization has expressed the concern of an upcoming post-antibiotic era, where otherwise 'simple' infections may become a real threat again [1]. Currently, in the USA, it is estimated that more than 35,000 deaths each year are a result of infection by multi-resistant bacteria [2]. Globally, the numbers are increasing, with a possibility of 10 million deaths per year by 2050 [3, 4]. Furthermore, little is invested in the development of new antibacterial agents by pharmaceutical companies, as the antimicrobial agent market is less profitable than other markets, such as chemotherapy [5]. Overall, due to the limited number of treatment possibilities in bacterial infections, antibiotic resistance is a real threat to humanity in the present and future to come.

Alternatives to antibiotics are likely to be needed to prevent this post-antibiotic era from happening. Next to other alternatives such as antibodies, probiotics, lysins, and immune stimulation, bacteriophage and bacteriophage-based products have shown potential as an option to combat the antimicrobial resistance problem [6-8]. Every year, there is an increasing focus on bacteriophage therapy, which is shown by the growing amount of citations per year from 1100 around 2015 to 1400 in 2017 [9]. From 2017 on, the Dutch program 'Dokters van Morgen' made several episodes on bacteriophages and its application around the world, leading to more attention for phages among the public in the Netherlands [10, 11]. Despite the recognition, more investment is needed in these therapies as the current knowledge about bacteriophage therapy is lacking. Moreover, a review written in 2016 predicted only a nine per cent chance for bacteriophage therapy to be implemented for the infections caused by *C. difficile* and *P. aeruginosa* by 2025 [6].

In this article, we will address the question of whether bacteriophage therapy can become an alternative to antibiotic therapy. Starting with the biology of bacteriophages, the history and the therapy will be elaborated on, ending with a review of the opportunities and concerns regarding the therapy. Do you think bacteriophage therapy is the alternative we need to treat bacterial infections in the future?

History of phages

In 1915, Twort was the first to describe the possibility of a virus that could infect bacteria, but unfortunately, he could not confirm this hypothesis [12]. Two years later, the term bacteriophage was confidently coined by D'Herelle, who found the kind of viruses described by Twort [13]. Developing phage therapy was started with much enthusiasm, but the enthusiasm calmed down after the discovery of antibiotics and their use in the Second World War [14]. In the years after the war, in countries in Eastern Europe, specifically the Soviet Union and Poland, phage therapy was further used and developed. Most of the current knowledge and experience with the therapy is associated with The Hirszfeld Institute in Poland and the Eliava Institute in Georgia [15]. The Hirszfeld Institute is mainly focused on an individualised approach, specific for the bacterium in the patient, and reported cure rates of around 40 per cent [14, 15]. On the other hand, the focus of the Eliava Institute is more on the development and use of phage cocktails [14, 15]. In Georgia, the phage therapy was part of the standard care long before double-blinded clinical trials were developed; therefore, the experiences in these countries are not well documented [14].

Biology of bacteriophages

Bacteriophages are viruses that infect and kill bacteria [15]. In every natural environment where a bacterial host exists, the corresponding bacteriophage is also present. The phages play an essential role in many biological processes, after all, they are the most plentiful organism on the planet [16]. Bacteriophages act on specific bacteria or a specific strain of a bacterium [15]. Thus, when a bacterium has multiple strains, various bacteriophages can act on it. There are two types of bacteriophages that infect bacteria via two distinct pathways. The virulent phages have a lytic cycle in which the phage attaches itself to the bacterium, inserts its genome, multiplies, and lyses the cell, releasing the new viruses [17]. This lytic cycle can be compared to the way viruses infect humans. On the other hand, the temperate phages initiate a lysogenic cycle starting with the phage being dormant as a prophage, replicating along with its host, and sometimes beginning a lytic cycle through a specific trigger

[17]. Considering that these temperate phages can be beneficial to the bacteria by helping to encode genes for virulence factors, they should currently not be used in phage therapy [18-20].

How to set up bacteriophage therapy

A multi-step process should be followed to arrange a suitable treatment for a patient experiencing a multi-resistant bacterial infection (Figure 1). The first step in the treatment of a patient with phage therapy is the selection of the bacteriophage [15]. Without the proper selection of the right phage, failure of therapy is very likely [20]. The choice of phage can be quite simple in the case of a mono-bacterial infection, as only a phage that infects that specific bacterium needs to be selected [20]. For more complex situations, phage cocktails or banks may be needed. Phage cocktails are formulations with two or more phage species that each target a specific species of bacteria [20]. Phage banks are a collection of phages against specific bacteria that are isolated and can be used when needed [7]. The advantage of phage cocktails is its efficacy as you hit the bacteria with multiple phages, whereas the advantage of banks is the specificity of the therapy [20]. These banks and cocktails are especially useful when bacteria become phage-resistant during the treatment with a specific phage [20, 21]. But where do the bacteriophages in these banks and cocktails come from?

That is where the next three steps in the development of a proper treatment come into play, starting with the isolation of the phages [15]. For the isolation of the phages, an environmental sample that is known to contain the bacterial host, like water, soil, or cattle faeces, is needed [20]. For example, phages have been isolated in cattle feedlots that infect *E. coli* and *Salmonella* spp. [22]. In this process, strains of bacteria need to be isolated that are identical (or at least as similar as possible) to the strains of bacteria that will be encountered in the patient [20]. The next step, after isolation, is the characterisation of the phages, which is done to make sure that the phages are active against the bacterial strains present in the patient. Subsequently, the representative sample of the phages used is characterised to ensure that the treatment is active against the bacterial strains in the patient [20]. The last step is the preparation of the phages, which comprises amplification and purification [20]. This includes a titre of the phage preparation to ensure appropriate dosing and purity. When this step is completed, the patient can be treated with the phages (Figure 1).

Bumps in the road to implementation

Considering that much is known about the biology of bacteriophages and the way to set up treatment, bacteriophage therapy shows high potential. Nevertheless, implementation in the standard care worldwide seems far from easy. The first challenge is the lack of sufficient information retrieved from the currently performed controlled clinical trials [23]. In the last few years, two main clinical trials were performed [24, 25]. The first one reported on the safety of phage therapy for treating venous leg ulcers and found no adverse effects [24]. The second trial demonstrated the efficacy and safety of its use in chronic otitis [25]. Currently, several clinical trials are registered to take place in the next few years [23]. Compared to other drugs, controlled clinical trials for bacteriophage therapy come with unique considerations, such as the dosage, host spectrum activity, and phage resistance. The issues regarding broad bacterial infectivity and phage resistance are already tackled with effective methods like broad host range phages or cocktails [21]. As for safety during the trials, the purity of the preparation should be sufficient. This is a major challenge for widespread use, as viruses can mutate during the amplification for *in vivo* use, possibly troubling the purity of the virus. Quality parameters for the engineering process of phage preparation are already formulated [26].

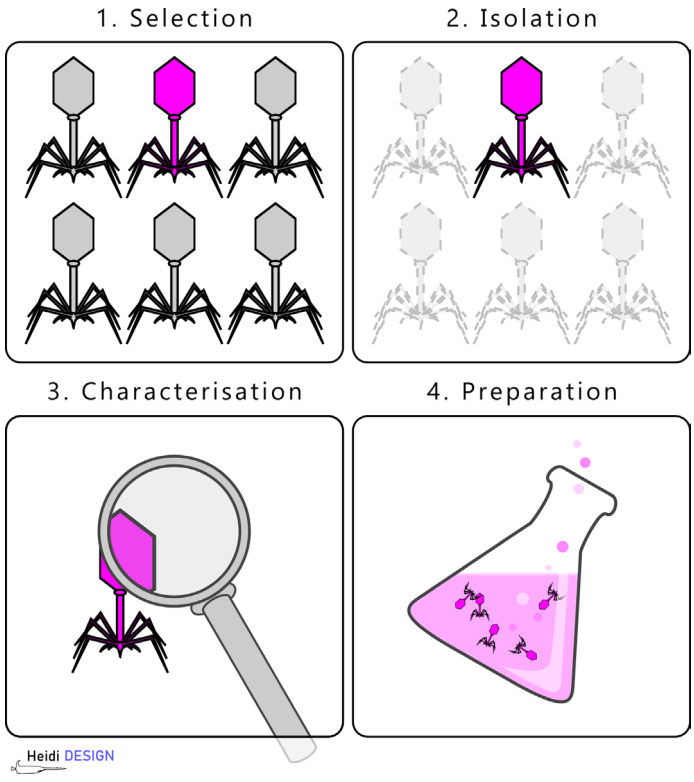


Figure 1: The development of bacteriophage therapy: a multi-step process
First the right bacteriophage has to be selected. After this selection, the phage should be isolated from an evironmental sample. Then, effectiveness against the bacterium is ensured and afterwards the phage preparation can be amplified and purified.

Outside of Georgia and Poland, no regulatory framework exists for the use of bacteriophages in the medical context [23]. In the literature, some researchers have suggested developing such a regulatory framework [27, 28]. Verbeken *et al.* analysed the stakeholder opinions and found that the stakeholders agreed about the need for a regulatory framework for the phage therapy; one for the properties and interactions of phages and one for the role of hospitals in the therapy [28]. Besides, a workshop with the European Medicines Agency acknowledged the need for a new regulatory framework as the current regulations are unsuitable for phages [29]. The magistral approach in Belgium is an example of a situation where political progression results in a framework for the use of phage therapy [30]. It centres on the preparation of tailored products that contain non-authorised ingredients (the phages) but have a certificate from a Belgian Approved Laboratory. These products can be used in Belgium to treat patients [31].

Although it has been approved in Belgium, the reaction of the general public on a possible widespread implementation of bacteriophage therapy is hard to predict. For example, vaccinations are not supported by everyone, often leading to discussion when new vaccinations enter the market. Injecting a living, functioning virus into the body of patients could lead to similar discussions. Would you inject yourself with a live virus?

Conclusion

Standing on the doorstep of the post-antibiotic era: does bacteriophage therapy bring light at the end of the tunnel? Indeed, bacteriophage therapy shows the potential to be one of the alternatives to antibiotics. These bacteria-

infecting viruses were discovered earlier than the antibiotics themselves, but the scientific Western world has slept on them for a long time. On the other hand, Eastern Europe, especially Poland, Georgia, and Russia, already has a lot of experience with treating patients with phage therapy. The engineering of phage therapy is known to be a multi-step process, comprising selection, isolation, characterisation, and preparation of the phages. However, the lack of clinical trials and a regulatory framework currently prevents bacteriophages from entering the drug market. Nevertheless, the magistral approach of Belgium shows the implementation of phage therapy is possible, giving us hope for a future with widespread bacteriophage use as a treatment for bacterial infections.

Acknowledgements

RAMS would like to thank Jelmer Raaijmakers, MSc, Department of Medical Microbiology, Radboudumc, Nijmegen, the Netherlands for providing the author of this article with feedback on this article.

References

1. Thomas, G. WHO's first global report on antibiotic resistance reveals serious, worldwide threat to public health. World Health Organization. (2014).
2. Cdc. Antibiotic resistance threats in the United States. U.S. Department of Health and Human Services, Atlanta. (2019).
3. Sugden, R., *et al.* Combatting antimicrobial resistance globally. *Nature microbiology* **1**, 16187 (2016).
4. O'neill, J. Antimicrobial Resistance: Tackling a crisis for the health and wealth of nations. (2014).
5. Projan, S.J. Why is big Pharma getting out of antibacterial drug discovery? *Current opinion in microbiology* **6**, 427-430 (2003).
6. Czaplewski, L., *et al.* Alternatives to antibiotics-a pipeline portfolio review. *The Lancet. Infectious diseases* **16**, 239-251 (2016).
7. Balogh, B., *et al.* Phage therapy for plant disease control. *Current pharmaceutical biotechnology* **11**, 48-57 (2010).
8. Kutter, E., *et al.* Phage therapy in clinical practice: treatment of human infections. *Current pharmaceutical biotechnology* **11**, 69-86 (2010).
9. Górski, A., *et al.* Phage Therapy: What Have We Learned? *Viruses* **10** (2018).
10. Dokters van Morgen. Bacteriofagen. (2020). Retrieved from: https://zorgnu.avrotros.nl/dossiers/item/bacteriofagen/#/ (Accessed: 15-10-2020)
11. Fagenbank. Bacteriofagen in de media. (2020). Retrieved from: https://www.fagenbank.nl/nederlands/in-de-media/ (Accessed: 15-10-2020)
12. Twort, F.W. AN INVESTIGATION ON THE NATURE OF ULTRA-MICROSCOPIC VIRUSES. *The Lancet* **186**, 1241-1243 (1915).
13. D'herelle, F. On an invisible microbe antagonistic toward dysenteric bacilli: brief note by Mr. F. D'Herelle, presented by Mr. Roux. 1917. *Research in microbiology* **158**, 553-554 (2007).
14. Abedon, S.T., *et al.* Phage treatment of human infections. *Bacteriophage* **1**, 66-85 (2011).
15. Kakasis, A. & Panitsa, G. Bacteriophage therapy as an alternative treatment for human infections. A comprehensive review. *International journal of antimicrobial agents* **53**, 16-21 (2019).
16. Keen, E.C. A century of phage research: bacteriophages and the shaping of modern biology. *BioEssays : news and reviews in molecular, cellular and developmental biology* **37**, 6-9 (2015).
17. Kutter, E.M. & Sulakvelidze, A. *Bacteriophages : biology and applications*, (Boca Raton (Fla.) : CRC Press, 2005).
18. Lin, D.M., *et al.* Phage therapy: An alternative to antibiotics in the age of multi-drug resistance. *World journal of gastrointestinal pharmacology and therapeutics* **8**, 162-173 (2017).
19. Clark, J.R. Bacteriophage therapy: history and future prospects. *Future Virology* **10**, 449-461 (2015).
20. Gill, J.J. & Hyman, P. Phage choice, isolation, and preparation for phage therapy. *Current pharmaceutical biotechnology* **11**, 2-14 (2010).
21. Goodridge, L.D. Designing phage therapeutics. *Current pharmaceutical biotechnology* **11**, 15-27 (2010).
22. Johnson, R.P., *et al.* Bacteriophages for prophylaxis and therapy in cattle, poultry and pigs. *Animal health research reviews* **9**, 201-215 (2008).
23. Furfaro, L.L., *et al.* Bacteriophage Therapy: Clinical Trials and Regulatory Hurdles. *Frontiers in cellular and infection microbiology* **8**, 376 (2018).
24. Rhoads, D.D., *et al.* Bacteriophage therapy of venous leg ulcers in humans: results of a phase I safety trial. *Journal of wound care* **18**, 237-238, 240-233 (2009).
25. Wright, A., *et al.* A controlled clinical trial of a therapeutic bacteriophage preparation in chronic otitis due to antibiotic-resistant Pseudomonas aeruginosa; a preliminary report of efficacy. *Clinical otolaryngology : official journal of ENT-UK ; official journal of Netherlands Society for Oto-Rhino-Laryngology & Cervico-Facial Surgery* **34**, 349-357 (2009).
26. Parracho, H.M., *et al.* The role of regulated clinical trials in the development of bacteriophage therapeutics. *Journal of molecular and genetic medicine : an international journal of biomedical research* **6**, 279-286 (2012).
27. Huys, I., *et al.* Paving a regulatory pathway for phage therapy. Europe should muster the resources to financially, technically and legally support the introduction of phage therapy. *EMBO reports* **14**, 951-954 (2013).
28. Verbeken, G., *et al.* Call for a dedicated European legal framework for bacteriophage therapy. *Archivum immunologiae et therapiae experimentalis* **62**, 117-129 (2014).
29. Pelfrene, E., *et al.* Bacteriophage therapy: a regulatory perspective. *The Journal of antimicrobial chemotherapy* **71**, 2071-2074 (2016).
30. Pirnay, J.P., *et al.* The Magistral Phage. *Viruses* **10** (2018).
31. Moelling, K., *et al.* A Wake-Up Call: We Need Phage Therapy Now. *Viruses* **10** (2018).

ZEBRAS OF MEDICINE

ANTI-NMDAR ENCEPHALITIS VS SCHIZOPHRENIA: A COMPARISON BETWEEN TWO CLINICAL REPRESENTATIONS

Elisah Geertman[1]

[1] Bachelor's student Medicine, Radboud university medical centre, Nijmegen, The Netherlands

Abstract

Background: At the onset of anti-N-Methyl-D-aspartate receptor (anti-NMDAR) encephalitis, an autoimmune disease with antibodies against the NMDAR, approximately 90% of patients experience psychiatric or behavioural symptoms, and in particular, psychosis. This makes it difficult to differentiate the condition from a primary psychiatric disease such as schizophrenia.

Objective: This review aims to delineate clinical signs that could enhance differentiation between anti-NMDAR encephalitis and schizophrenia and compare both conditions in terms of diagnosis and treatment.

Discussion: When comparing anti-NMDAR encephalitis with schizophrenia, a difference can be seen in the expression of positive symptoms, e.g. hallucinations and delusions, and negative symptoms, such as apathy and poverty of speech. In schizophrenia, positive symptoms are more frequent than negative symptoms at disease onset, whereas both positive and negative symptoms are usually present at the onset of anti-NMDAR encephalitis. Symptoms generally also have a more rapid progression in anti-NMDAR encephalitis. Other symptoms exclusively seen in anti-NMDAR encephalitis are seizures, decreased level of consciousness, dyskinesias, and autonomic instability.

Conclusion: It is challenging to distinguish anti-NMDAR encephalitis from a primary psychiatric disorder like schizophrenia, especially in early stages. However, during the subacute onset of anti-NMDAR encephalitis, several distinctive clinical warning signs emerge, such as decreased level of consciousness, focal neurological deficits, and epileptic seizures. These warning signs should lead to consultation with a neurologist where detection of IgG antibodies against the NMDAR in the cerebrospinal fluid is crucial to prevent misdiagnosis. In this way, diagnostic accuracy can be enhanced, resulting in more adequate patient management.

KEYWORDS: Schizophrenia, Autoimmune encephalitis, Autoimmune psychosis

Anti-N-Methyl-D-aspartate receptor (anti-NMDAR) encephalitis is a form of autoimmune encephalitis that was first described in 2007 [1]. The pathophysiology of anti-NMDAR encephalitis involves an autoimmune reaction with antibodies against one of the subunits of the NMDAR [1]. This receptor plays an essential role in neuroplasticity, synaptic transmission, memory, learning, and human behaviour [1]. At the onset of anti-NMDAR encephalitis, approximately 90% of patients are reported to experience psychiatric or behavioural symptoms, making it challenging to distinguish the condition from a primary psychiatric disease [2]. These prominent psychiatric manifestations that often occur before the onset of neurological symptoms can result in misdiagnosis, which delays diagnosis and treatment [3]. In the early stages of the disease, patients with anti-NMDAR encephalitis have been misdiagnosed with several psychotic disorders, including schizophrenia, schizoaffective disorder, bipolar disorder, and depression [1]. Schizophrenia can be characterised by delusions, hallucinations, disorganisation, formal thinking changes, and catatonia, typically occurring for the first time during adolescence and early adulthood [4]. Early recognition of anti-NMDAR encephalitis is essential, considering that this is a treatable neurological disorder that can have a severe clinical course when no treatment is applied. This review aims to delineate the characteristic properties of anti-NDMDAR encephalitis and schizophrenia.

Clinical presentation

Schizophrenia

The mean age of schizophrenia presentation is 25-35 years in women and 18-25 years in men, with a female to male ratio of 0.92:1 [2]. Patients with schizophrenia generally present with symptoms that can be classified under four distinct areas of psychopathology: positive symptoms, negative symptoms, cognitive impairment, and mood and anxiety symptoms [5]. Positive symptoms generally appear in adolescence or early adulthood and mark the formal onset of schizophrenia [4]. These include reality distortions as hallucinations and delusions, as well as disorganisation in behaviour, thinking, and speech [4]. Auditory hallucinations, such as hearing voices, are the most common [4].

While positive symptoms represent an exaggeration of normal processes, negative symptoms embody an absence or diminution of normal processes. These symptoms involve a reduction or loss of affective and conative functions, including poverty of speech, inability to experience pleasure, lack of initiative and interest, and loss of motivation. Positive symptoms tend to relapse and remit, whereas negative and cognitive symptoms are often chronic [5]. Impairments in cognition can include deficits in attention, memory, and executive functions [5]. Mood and

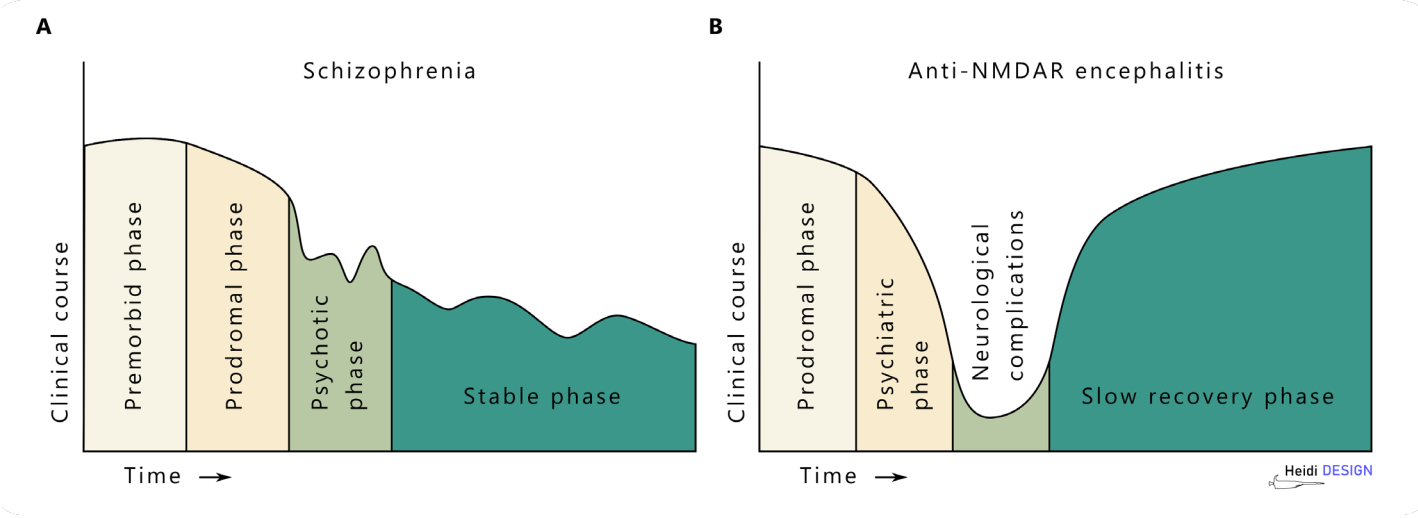


Figure 1: Clinical course of schizophrenia and NMDAR-encephalitis

anxiety disorders, such as depression, are also common in patients with schizophrenia [5]. In addition, there are several physical manifestations associated with schizophrenia, including neurological disturbances, catatonia, and metabolic disturbances [4].

The clinical course of schizophrenia involves four different phases: the premorbid phase, the prodromal phase, the psychotic phase and the stable phase (Figure 1A) [4]. Although useful in some respects, it is important to note that the differentiation between these phases is imprecise [4]. Among patients, there is variation in illness progression and over 50% of individuals with attenuated psychotic symptoms, indicative of the prodromal phase of schizophrenia, eventually do not develop schizophrenia [4]. To summarise, the typical clinical course of schizophrenia exists out of four different phases with different positive, negative, cognitive, and mood and anxiety symptoms throughout [4].

Anti-NMDAR encephalitis

The median age at anti-NMDAR encephalitis onset is 21 years, but cases have been described in patients ranging from eight months to 85 years [2]. With a female to male ratio of 8:2, anti-NMDAR encephalitis is a female predominant disease which is also associated with a number of malignancies, of which ovarian teratomas are the most common [2]. In 59% of cases, the diagnosis was associated with ovarian teratomas [6].

Before the onset of neurological manifestations, prodromal symptoms such as fever, headache, nausea, vomiting, diarrhoea, and flu-like symptoms appear in approximately 70% of patients with anti-NMDAR encephalitis [7]. At an early stage, patients can display behavioural complaints, psychosis, delusions, hallucinations, and paranoia, which are often accompanied by memory deficits and language disturbances [2, 7]. Movement disorders are also common in patients with anti-NMDAR encephalitis [2, 7-9]. Ultimately, patients may progress to catatonia, a psychomotor syndrome that occurs in over 10% of patients with psychiatric illnesses, or mutism, followed by a decreased level of consciousness and autonomic instability [7-9].

Psychiatric symptoms and seizures are more common in adults compared to children, with 16% of female patients and 34% of male patients experiencing seizures [7]. Children, on the other hand, often present with behavioural symptoms and movement disorders [7]. Overall, the most common symptom in anti-NMDAR encephalitis is agitation, followed by psychotic symptoms (54%) and catatonia in 42% of adults and 35% of children [8]. In short, the typical clinical course of anti-NMDAR encephalitis can be divided into a prodromal period, a psychiatric period, a period of neurological complications and finally, a slow recovery phase with prolonged deficits which can last for months or years (Figure 1B) [2, 9]. Although the majority of patients recover well, anti-NMDAR encephalitis can result in residual cognitive deficits [9].

Differences in clinical presentation

The prominent psychiatric and behavioural symptoms featured in the majority of patients with anti-NMDAR encephalitis at disease onset can make it difficult to distinguish the condition from a primary psychiatric disease [2]. The acronym "SEARCH For NMDAR-A" covers a combination of diagnostic clues that should raise suspicion for anti-NMDAR encephalitis in patients with new-onset psychiatric symptoms (Table 1) [2].

When comparing the clinical presentation of anti-NMDAR encephalitis with that of schizophrenia, differences can be seen in the expression of positive and negative symptoms [2]. In schizophrenia, positive symptoms are more frequent than negative symptoms at disease onset [2]. In anti-NMDAR encephalitis, positive and negative symptoms are usually present at the onset and symptoms develop in days or weeks [2]. The rapid onset of the disease is also in contrast with primary psychiatric disorders, in which symptoms usually have a more graduate progression, or, in case of acute psychosis, the onset is generally preceded by behavioural changes [2]. Other symptoms that differ from those seen in schizophrenia are seizures, decreased level of consciousness, dyskinesias, and autonomic instability [2]. These differences, as well as the diagnostic clues included in the mentioned acronym, might be useful to keep in mind when evaluating a patient presenting with psychiatric symptoms.

Diagnosis

A specific diagnosis of schizophrenia is one of exclusion and is only made after several months of observation using ICD-10 or DSM-5 criteria [11]. Delusions, hallucinations, disorganised speech or behaviour, and negative symptoms are characteristic symptoms of the disorder, and according to the DSM-5, at least two of these symptoms should be present to make the diagnosis [12]. These symptoms should be linked with social dysfunction for a minimum duration of six months [12]. Further diagnostic criteria incorporate that another diagnosis that would better explain the presentation should be absent [12].

With regard to recognising possible anti-NMDAR encephalitis in patients with psychotic symptoms, the following clinical warning signs could be helpful: subacute onset, defined as rapid progression within three months despite pharmacotherapy, decreased consciousness level, memory deficits or disorientation, catatonia, speech dysfunction (aphasia or dysarthria), abnormal postures or movements (dystonia or dyskinesia), focal neurological deficits, autonomic dysfunction, headache, hyponatremia, other autoimmune diseases, and epileptic seizures [2, 8, 13].

Table 1: Diagnostic clues for anti-NMDAR encephalitis in patients with new-onset psychiatric symptoms (SEARCH For NMDAR-A) [2]

S	Sleep dysfunction	Severe sleeplessness is more common than narcolepsy at the onset, whereas narcolepsy is more frequent during the phase of recovery. This is sometimes associated with excessive eating and hypersexuality.
E	Excitement, disinhibition, or manic behaviour alternating with depressive behaviour	Manic and bizarre behaviour, hypersexuality, or wandering are frequent at disease onset, whereas depression and suicidality are less common.
A	Agitation or aggression	Similar in children and adults.
R	Rapid onset	Symptoms develop in days or weeks. By contrast, symptoms have a slower progression in primary psychiatric diseases. In case of acute psychosis, behavioural changes are often seen before the onset.
C	Children and young adult predominance	Median age at disease onset is 21 years, and the disease is more common in women.
H	History of psychiatric disease absent	Previous episodes of psychosis or behavioural change are often caused by anti-NMDAR encephalitis.
F	Fluctuating catatonia	Catatonia can alternate with episodes of agitation.
N	Negative and positive symptoms at presentation	Both positive and negative symptoms are usually present at the onset.
M	Memory deficit	Most patients do not remember a large period of their disease course after they have recovered.
D	Decrease of verbal output or mutism	Decrease of verbal output occurs rapidly, particularly in children, and can be preceded by pressured speech.
A	Antipsychotic intolerance	Applicable to typical and atypical antipsychotics.
R	Rule out neuroleptic malignant syndrome	Patients considered to have a primary psychiatric disease and are treated with neuroleptics, can be misdiagnosed with neuroleptic malignant syndrome instead of anti-NMDAR encephalitis.
A	Antibodies and additional paraclinical tests (EEG), MRI, or CSF)	NMDAR antibodies are always present in CFS and 80% of patients have pleocytosis.

In order to aid the diagnosis of anti-NMDAR encephalitis, a set of diagnostic criteria was developed in 2016 (Table 2) [2]. Although these criteria may facilitate early diagnosis and treatment, demonstration of IgG antibodies against a specific subunit of the NMDAR is the only test, for now, that can confirm the diagnosis [2]. Considering that NMDAR antibodies are always present in cerebrospinal fluid (CSF) at time of diagnosis and 80% of patients have pleocytosis, which is an increased cell count, these antibodies have higher specificity and sensitivity compared to serum antibodies [2]. However, it is recommended to test both CSF and serum to avoid false-negative or false-positive results [14].

Electroencephalography (EEG) can be helpful to distinguish encephalitis from a primary psychiatric disorder since 90% of patients with anti-NMDAR encephalitis have evidence of non-specific slowing at some point during the disease [6, 13]. The most frequent abnormal EEG finding is slow-wave with or without epileptic features [6]. As for brain imaging techniques, MRI only shows abnormalities in 33% of the cases [14]. Due to the higher incidence of malignancies in patients with anti-NMDAR encephalitis, tumour screening with CT, MRI, and abdominal and transvaginal ultrasound is strongly recommended [15].

All in all, in the case of anti-NMDAR encephalitis, detection of IgG antibodies in CSF or plasma is the gold standard to obtain the diagnosis, but other diagnostic measures such as EEG or MRI may be used to exclude other causes and support the diagnosis [13]. For schizophrenia, the diagnosis is made based on clinical presentation and prolonged evaluation using ICD-10 or DSM-5 criteria [11].

Treatment

Even though schizophrenia and anti-NMDAR encephalitis share a number of clinical features, there are significant differences in terms of treatment. Schizophrenia requires lifelong treatment and management. A combination of pharmacologic agents and psychological interventions is recommended for first-episode psychosis, acute exacerbations, and prevention of relapse of psychosis [16]. The first-line pharmacological treatment of acute psychosis consists of second-generation oral antipsychotics, such as haloperidol or olanzapine. [17]. Sometimes an antipsychotic of this type is commenced in combination with adjunctive medication, for example, a benzodiazepine or antidepressant, when clinically indicated [16]. Examples of psychosocial interventions that are proven to be effective in the management of schizophrenia are cognitive behavioural therapy, group therapy, and social skills training [18]. For adult patients who do not respond to pharmacological treatment, electroconvulsive therapy may be considered [17].

For anti-NMDAR encephalitis, immunotherapy is the primary treatment [1]. First-line immunotherapy includes high-dose steroids, such as methylprednisolone, intravenous gamma globulin, and plasma exchange [1]. If a tumour is present, tumour resection is also a part of first-line treatment [1]. Second-line immunotherapy, such as rituximab or cyclophosphamide, can be used when patients are unresponsive to first-line immunotherapy, which is the case in 50% of the patients [1]. Psychiatric symptoms often require additional treatment through multiple psychotropic drugs [1]. Anti-NMDAR encephalitis can be lethal but often has a good prognosis if diagnosed and treated quickly. In the case of schizophrenia, delayed treatment may increase the risk of brain volume loss [16]. Thus, although schizophrenia is a chronic disease and anti-NMDAR encephalitis is a

Table 2: Diagnostic criteria for anti-NMDAR encephalitis [2]

Probable anti-NMDAR encephalitis:	
A.	Rapid onset (<3 months) of at least four of the six following significant groups of symptoms (or three if the patient has an underlying tumour): 1. Abnormal (psychiatric) behaviour or cognitive dysfunction 2. Speech dysfunction (pressured speech, verbal reduction or mutism) 3. Seizure 4. Movement disorder, dyskinesias or rigidity/abnormal postures 5. Decreased level of consciousness 6. Autonomic dysfunction or central hypoventilation
B.	At least one of the following laboratory study results: 1. Abnormal EEG (focal or diffuse slow or disorganized activity, epileptic activity, or extreme delta brush) 2. CSF with pleocytosis or oligoclonal bands
C.	Reasonable exclusion of other disorders, such as CNS infection, herpes simplex virus encephalitis, epileptic disorders, or known psychiatric disease.
Definite anti-NMDAR encephalitis:	
	Presence of at least one of the six major groups of symptoms (A), and IgG anti-GluN1 antibodies, after reasonable exclusion of other disorders (C).

reversible disorder, it is crucial for both conditions to start treatment as soon as possible.

Conclusions

It is challenging to distinguish anti-NMDAR encephalitis from a primary psychiatric disorder like schizophrenia, especially in early stages. Symptoms seen in anti-NMDAR encephalitis that differ from those seen in schizophrenia include seizures, decreased level of consciousness, dyskinesias, and autonomic instability [2].

Overall, clinical warning signs of possible anti-NMDAR encephalitis in patients with psychotic symptoms include the following: subacute onset, memory deficits or disorientation, catatonia, speech dysfunction (aphasia or dysarthria), abnormal postures or movements (dystonia or dyskinesia), focal neurological deficits, autonomic dysfunction, headache, hyponatremia, other autoimmune diseases, and most importantly epileptic seizures [2, 8, 13]. These warning signs should lead to consultation with a neurologist. Determination of IgG antibodies against the NMDAR in CSF is crucial to prevent misdiagnosis in anti-NMDAR encephalitis and should be performed in every patient presenting with psychiatric symptoms in combination with one or more of the mentioned warning signs [2]. In this way, diagnostic accuracy can be enhanced, resulting in more adequate patient management.

Acknowledgements

RAMS would like to thank S. Lassche, MSc, from the Department of Neurology, Radboudumc, Nijmegen, The Netherlands, for proofreading this article and providing feedback.

References

1. Huang, Q., *et al.* Anti-N-methyl-D-aspartate receptor encephalitis: A review of pathogenic mechanisms, treatment, prognosis. *Brain Research* **1727**, 146549 (2020).
2. Dalmau, J., *et al.* An update on anti-NMDA receptor encephalitis for neurologists and psychiatrists: mechanisms and models. *The Lancet Neurology* **18**, 1045-1057 (2019).
3. Gibson, L.L., *et al.* The Psychiatric Phenotype of Anti-NMDA Receptor Encephalitis. *The Journal of Neuropsychiatry and Clinical Neurosciences* **31**, 70-79 (2019).
4. Tandon, R., *et al.* Schizophrenia, "just the facts" 4. Clinical features and conceptualization. *Schizophrenia Research* **110**, 1-23 (2009).
5. Owen, M.J., *et al.* Schizophrenia. *Lancet* **388**, 86-97 (2016).
6. Barry, H., *et al.* Anti-N-methyl-d-aspartate receptor encephalitis: review of clinical presentation, diagnosis and treatment. *BJPsych Bull* **39**, 19-23 (2015).
7. Dutra, L.A., *et al.* Autoimmune encephalitis: a review of diagnosis and treatment. *Arquivos de Neuro-Psiquiatria* **76**, 41-49 (2018).
8. Herken, J. & Prüss, H. Red Flags: Clinical Signs for Identifying Autoimmune Encephalitis in Psychiatric Patients. *Frontiers in Psychiatry* **8**(2017).
9. Finke, C., *et al.* Cognitive deficits following anti-NMDA receptor encephalitis. *J Neurol Neurosurg Psychiatry* **83**, 195-198 (2012).
10. Kayser, M.S. & Dalmau, J. Anti-NMDA receptor encephalitis, autoimmunity, and psychosis. *Schizophrenia Research* **176**, 36-40 (2016).
11. Soares-Weiser, K., *et al.* First rank symptoms for schizophrenia. *Cochrane Database Syst Rev* **1**, CD010653-CD010653 (2015).
12. Anonymous. *Schizophrenia Spectrum and Other Psychotic Disorders*. in Diagnostic and Statistical Manual of Mental Disorders, Vol. (ed.^(eds. https://dsm-psychiatryonline-org.ru.idm.oclc.org/doi/full/10.1176/appi.books.9780890425596.dsm02
13. Steiner, J., *et al.* Autoimmune encephalitis with psychosis: Warning signs, step-by-step diagnostics and treatment. *The World Journal of Biological Psychiatry* **21**, 241-254 (2020).
14. Endres, D., *et al.* Autoimmune encephalitis as a differential diagnosis of schizophreniform psychosis: clinical symptomatology, pathophysiology, diagnostic approach, and therapeutic considerations. *European Archives of Psychiatry and Clinical Neuroscience* (2020).
15. Amugoda, C., *et al.* Anti-NMDAR Encephalitis: Higher Suspicious Needed for Earlier Diagnosis (Case Report, Literature Review and Diagnostic Criteria). *Case Reports in Neurological Medicine* **2019**, 1-5 (2019).
16. Kahn, R.S., *et al.* Schizophrenia. *Nat Rev Dis Primers* **1**, 15067 (2015).
17. Keepers, G.A., *et al.* The American Psychiatric Association Practice Guideline for the Treatment of Patients With Schizophrenia. *American Journal of Psychiatry* **177**, 868-872 (2020).
18. Grover, S., *et al.* Clinical Practice Guidelines for Management of Schizophrenia. *Indian Journal of Psychiatry* **59**, 19-33 (2017).

DYSLIPIDEMIA AND HYPERGLYCEMIA IN PSORIATIC INPATIENTS

Bojan Popchanovski[1]

[1] Master's student Medicine, University Ss. Cyril and Methodius, Skopje, Republic of Macedonia, participant Summer School

Abstract

Clinical Study

Background: Psoriasis is a chronic cutaneous T-cell mediated disease, which has been associated with many comorbidities, such as metabolic disorders. Specific abnormalities include dyslipidemia, insulin resistance, obesity, and metabolic syndrome, many of which are themselves risk factors for other diseases. The goal of this study was to evaluate the presence of dyslipidemia and hyperglycemia in patients with psoriasis.

Methods: We compared 48 inpatients with plaque psoriasis aged 29-79, hospitalised between March 2018 and February 2019, to 48 age- and gender-matched controls. We evaluated dyslipidemia and hyperglycemia using enzymatic methods as part of a standard blood test or medication history indicative of ongoing treatment of dyslipidemia and/or hyperglycemia. Hypertension was evaluated by registering blood pressure greater than 140/90 mmHg or ongoing antihypertensive treatment. Smoking habits were also noted.

Results: There were statistically significant differences between psoriasis patients and controls for elevated total cholesterol (p=0.028), elevated low-density lipoprotein (LDL) (p=0.015), hypertriglyceridemia (p=0.006), and hyperglycemia (p=0.021). The two groups had statistically insignificant differences for lowered high-density lipoprotein (p=0.084), hypertension (p=1), and smoking (p=0.836).

Conclusion: Hypertriglyceridemia, hyperglycemia, and elevated LDL cholesterol were found to be more prevalent in the group containing psoriatic patients compared to the control group. This indicates that further investigation of metabolic abnormalities should be conducted in psoriatic patients, which could greatly benefit from early treatment of the aforementioned underlying conditions.

KEYWORDS: Psoriasis, Inpatients, Metabolic syndrome, Dyslipidemias, Hyperglycemia

Psoriasis is a chronic immune-mediated skin disorder, with a prevalence of 2% [2]. Tumor necrosis factor alpha, interferon alpha, interleukin 23, and T-helper 17 cells play an important role in the pathogenesis of psoriasis [3]. Recent evidence suggests that metabolic abnormalities are present in the milieu of chronic inflammation, as in the case of rheumatological diseases [4]. Chronic inflammation is thought to cause cytokine-induced changes in glucose and lipoprotein metabolism, alluding to a similar situation that happens in the case of insulin resistance caused by cytokines secreted by adipose tissue [5, 6].

The amount of data on the effect of psoriasis on metabolism is increasing, but various results have been reported. For triglyceride levels, there are studies that found increased levels as well as statistically insignificant changes [7-13]. There are studies that associated psoriasis with higher, and others with normal LDL (low-density lipoprotein) cholesterol levels [7-9, 14-18]. As for HDL (high-density lipoprotein) cholesterol, there are studies that associated psoriasis with lower HDL levels and studies that did not make that association [7, 9, 14-20]. A correlation was reported between psoriasis and diabetes mellitus in some studies, and in other studies, no such correlation was made [7, 8, 15, 19, 21-25]. Results on hypertension and psoriasis were also conflicting, as there are studies that established a link and studies that did not [16, 21].

Quantitative and qualitative changes in lipoprotein metabolism, caused by chronic inflammation, may be of potential clinical significance in patients with a high risk of cardiovascular comorbidity. This study was conducted to examine the correlation between psoriasis and abnormal glucose and lipid metabolism.

Methods

This retrospective study took into account 48 inpatients (27 males, 21 females) with psoriasis vulgaris (placata and nummularis type) aged 29-79, hospitalised in the University Clinic of Dermatology at the Medical Faculty in Skopje, between March 2018 and February 2019. Data were derived from the clinic's inpatient medical records. Psoriatic inpatients that had pustular psoriasis, psoriatic arthritis, erythrodermia, prior systemic treatment for psoriasis, concomitant tumours, chronic lung, heart, kidney, and rheumatological diseases were excluded from the study. These 48 inpatients were paired with another 48 inpatients, matched for age (±1 year) and gender, hospitalised within the same timeframe, and in the same clinic. The exclusion criteria were the same for this group. The diagnoses of the control group inpatients were the following (Figure 1): urticaria acuta (23), reactio anaphylactica (5), reactio allergica post ictus ab insectis (4), erythema multiforme (3), eczema chronicum (3), oedema Quincke faciei (2), erysipelas (2), vasculitis (2) dermatitis arteficialis (1), Linear IgA bullous dermatosis (1), lichen ruber planus (1), and ulcera crurum (1).

The variables of interest were triglyceridemia, LDL cholesterol, HDL cholesterol, glycemia, blood pressure, and smoking habits. Lipid parameters and blood pressure were evaluated according to cutoff values recommended by the National Cholesterol Education Program's Adult

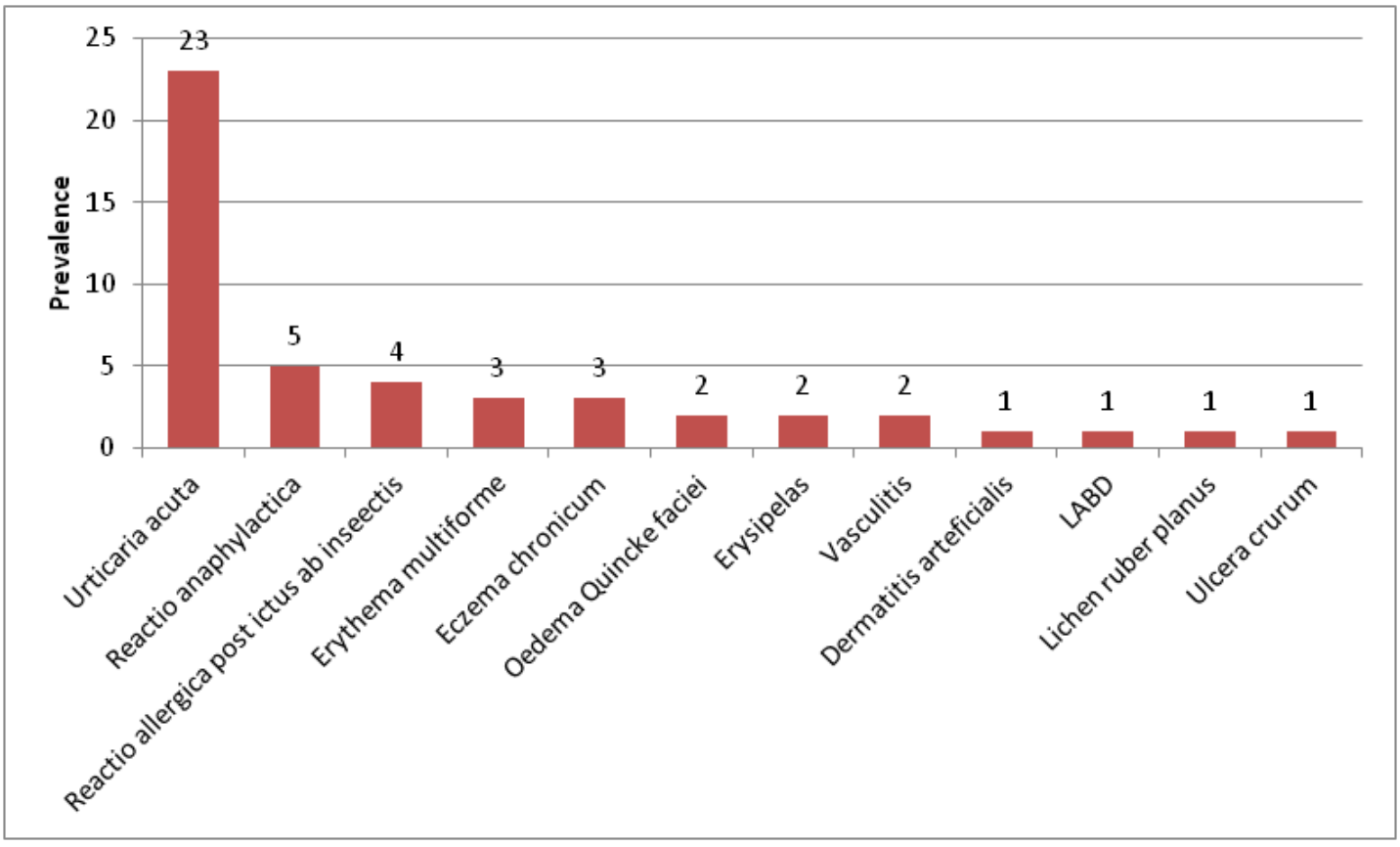


Figure 1: The diagnoses of the inpatients in the control group

Treatment Panel III, or ongoing antilipidemic and/or antihypertensive treatment according to the patient's medical history. These cutoff values were: 1.7 mmol/L for triglycerides, 3.3 mmol/L for LDL cholesterol, 1.0 mmol/L for HDL cholesterol, 140/90 mmHg for blood pressure. Glycemia was evaluated using the cutoff value of 6.1 mmol/L, recommended by the World Health Organization, or ongoing antidiabetic treatment [26]. Smoking habits were evaluated using two categories: patients who are non-smokers, and patients who are currently smoking or have smoked in the past. Glycemia and lipid parameters were measured using enzymatic methods. Blood pressure was measured with a standard mercury sphygmomanometer. RStudio was used to perform a Student's t-test and calculate the odds ratio with a 95% confidence interval.

Results

Among the 48 psoriatic patients, seven were aged between 29-39, 12 were aged between 40-49, 11 were aged between 50-59, 16 were aged between 60-69, and two were aged between 70-80. Identical distributions were present in the control group. In the psoriasis group, 14 patients (29.17%) had hyperglycemia, compared to five (10.42%) in the control group (p=0.021, OR 3.54, 95%CI 1.16-10.81). Hypertriglyceridemia was noted in 16 psoriatic patients (33.33%), and in five patients (10.42%) in the control group (p=0.006, OR 4.30, 95%CI 1.43-12.96). LDL cholesterol was increased in 16 psoriatic patients (33.33%), compared to 6 (12.5%) control patients, (p=0.015, OR 2.14, 95%CI 0.50-9.12). The differences between the psoriasis group and the control group were statistically insignificant for the remaining parameters. Ten (20.83%) psoriatic inpatients had lowered HDL cholesterol, compared to four (8.33%) control patients (p=0.084, OR 2.89, 95%CI 0.84-9.98). In the psoriasis group, 19 patients (39.58%) reported to have smoked or were current smokers, compared to 16 (33.33%) in the control group (p=0.836, OR 1.31, 95%CI 0.57-3.02). Finally, 16 patients in each group were found to have hypertension (p=1, OR 1.00, 95%CI 0.43-2.34) (Table 1).

Discussion

Despite the conflicting findings of the current body of research on this topic, there is a complex pathophysiological explanation for the quantitative and qualitative changes in the case of rheumatological diseases, which may also be true for psoriasis. Proinflammatory cytokines released during the course of these diseases change many aspects of lipid metabolism, such as increased very-low-density lipoproteins and triglyceride levels via increased hepatic fatty acid synthesis, decreased hepatic fatty acid oxidation, and increased adipose tissue lipolysis. This ultimately contributes to the increase of triglyceride content in LDL and HDL particles, which subsequently leads to the formation of small dense LDL particles. These particles are more atherogenic as a result of their high susceptibility to oxidation, high affinity for intra-arterial proteoglycans, and decreased clearance due to reduced affinity for LDL receptors. Additionally, lipoprotein lipase activity is reduced, which further reduces the clearance of LDL particles [4].

HDL particles are also subject to change in an inflammatory milieu, which equates to reverse cholesterol transport being severely impacted as a result. Apolipoprotein A-1 (Apo A-1) clearance is increased due to decreased synthesis and increased breakdown in the kidneys, which both lead to a lower affinity of Apo A-1 for HDL particles. Serum amyloid A, an acute-phase protein generated during inflammation, binds to HDL particles, which lowers the affinity of Apo A-1 for its receptor, and increases the clearance of HDL particles. Cholesterol ester transfer protein and lecithin-cholesterol acyltransferase levels are decreased, which lead to decreased cholesterol transport from HDL particles and decreased cholesterol ester

formation, respectively. Certain phospholipid and cholesterol membrane transport proteins, such as ATP-binding cassette transporter (ABC) A1, ABCG1, and scavenger receptor B1, have reduced activity, contributing to decreased hepatocyte uptake and decreased efflux from macrophages. Finally, lipoprotein (a) is generated, which has a high atherogenic potential [4]. This evidence of qualitative changes in lipoproteins suggests that perceived normal lipid levels may not be enough to exclude abnormalities in lipid metabolism.

The inflammatory pathogenesis of psoriasis suggests that, skin and joint lesions aside, many more less visible metabolic effects may be present. Psoriasis causes slight but clinically actionable alterations in certain metabolic parameters, which are relevant in terms of cardiovascular comorbidity.

This study could be improved by increasing the sample size to increase the accuracy of the data and to narrow down the confidence intervals. An important drawback represents its retrospective design. The data gathered were only the parameters that are measured during a routine examination. Additional useful parameters such as the Psoriasis Area and Severity Index and highly sensitive quantification of C-reactive protein to determine the extensiveness of the psoriatic lesions and the cardiovascular risk, respectively, could be measured and tested more appropriately in a case-control scenario.

Another aspect not covered in this study is disease progression. Our results are only indicative of one point in time, and the history of disease progress and treatment for each individual patient is unknown. Five of the previously mentioned studies stated that their objective was to determine the prevalence specifically of metabolic syndrome in psoriatic patients [15, 19, 21, 23, 24, 27]. Four of them associated psoriasis with metabolic syndrome, and one found no such link [15, 19, 21, 23, 24]. One of these previously mentioned studies established a dose-response relationship between the severity of psoriasis and the prevalence of metabolic syndrome, while another disproved that [24, 27]. One meta-analysis, taking 12 studies into account, also established a dose-response relationship [5]. These diverse findings pertaining to the metabolic syndrome, combined with the aforementioned diverse results on individual metabolic parameters, indicate that many other factors, such as the age of onset, duration, disease severity, and treatment, may play a role in terms of the order in which metabolic changes appear, and in the way they evolve over time.

Conclusion

The inflammatory pathogenesis of psoriasis suggests that skin and joint lesions aside, many more less visible metabolic effects may be present. Psoriasis causes slight but clinically actionable alterations in certain metabolic parameters, which are relevant in terms of cardiovascular comorbidity.

Acknowledgements

RAMS would like to thank Margareta Balabanova-Stefanova, PhD, University Ss. Cyril and Methodius, Skopje, Republic of Macedonia, for providing the author of this article with feedback.

Disclaimer

This article written by Bojan Popchanovski and Margareta Balabanova-Stefanova has been successfully published in the International Journal of Medical Students in December 2019 [1].

References

1. Popchanovski, B. & Balabanova-Stefanova, M. Dyslipidemia and Hyperglycemia in Psoriatic Inpatients. *International Journal of Medical Students* **7**, 62-65 (2019).
2. Christophers, E. Psoriasis – epidemiology and clinical spectrum. *Clinical and Experimental Dermatology* **26**, 314-320 (2001).
3. Boehncke, W.-H. & Schön, M.P. Psoriasis. *The Lancet* **386**, 983-994 (2015).

Table 1: Quantitative outcomes between the intervention group and the control group

Parameter	Psoriasis	Controls	p-value	OR (95% CI)
Mean age	52,92	52,73	/	/
Sex (male/female)	27/21	27/21	/	/
Smokers	19	16	0,836	1.31 (0.57-3.02)
↑ gly	14	5	0,021	3.54 (1.16-10.81)
↑ TAG	16	5	0,006	4.30 (1.43-12.96)
↓ HDL	10	4	0,084	2.89 (0.84-9.98)
↑ LDL	16	6	0,015	2.14 (0.50-9.12)
↑ BP	15	16	1	1.00 (0.43-2.34)

Legend: OR - odds ratio, CI - confidence interval, ↑gly - hyperglycemia, ↑TAG - elevated triglycerides, ↓HDL - lowered HDL cholesterol, ↑LDL - elevated LDL cholesterol, ↑BP - hypertension

4. Feingold, K.R. & Grunfeld, C. *The Effect of Inflammation and Infection on Lipids and Lipoproteins*, (MDText.com, Inc., South Dartmouth (MA), 2000).
5. Armstrong, A.W.*, et al.* Psoriasis and metabolic syndrome: A systematic review and meta-analysis of observational studies. *Journal of the American Academy of Dermatology* **68**, 654-662 (2013).
6. Shoelson, S.E.*, et al.* Inflammation and insulin resistance. *The Journal of Clinical Investigation* **116**, 1793-1801 (2006).
7. Seçkin, D.*, et al.* Are lipoprotein profile and lipoprotein (a) levels altered in men with psoriasis? *Journal of the American Academy of Dermatology* **31**, 445-449 (1994).
8. Reynoso-Von Drateln, C.*, et al.* Lipid profile, insulin secretion, and insulin sensitivity in psoriasis. *Journal of the American Academy of Dermatology* **48**, 882-885 (2003).
9. Piskin, S.*, et al.* Serum Lipid Levels in Psoriasis. *Yonsei Med J* **44**, 24-26 (2003).
10. Langan, S.M.*, et al.* Prevalence of metabolic syndrome in patients with psoriasis: a population-based study in the United Kingdom. *The Journal of investigative dermatology* **132**, 556-562 (2012).
11. Nisa, N. & Qazi, M.A. Prevalence of metabolic syndrome in patients with psoriasis. *Indian journal of dermatology, venereology and leprology* **76**, 662-665 (2010).
12. Asha, K.*, et al.* Dyslipidaemia & oxidative stress in patients of psoriasis: Emerging cardiovascular risk factors. *The Indian journal of medical research* **146**, 708-713 (2017).
13. Gisondi, P.*, et al.* Psoriasis and the metabolic syndrome. *Clinics in dermatology* **36**, 21-28 (2018).
14. Solak Tekin, N.*, et al.* Accumulation of Oxidized Low-Density Lipoprotein in Psoriatic Skin and Changes of Plasma Lipid Levels in Psoriatic Patients. *Mediators of Inflammation* **2007**(2007).
15. Damevska, K.*, et al.* Metabolic syndrome in untreated patients with psoriasis: case-control study. *JDDG: Journal der Deutschen Dermatologischen Gesellschaft* **11**, 1169-1175 (2013).
16. Miao, C.*, et al.* Obesity and dyslipidemia in patients with psoriasis: A case-control study. *Medicine (Baltimore)* **98**, e16323-e16323 (2019).
17. Asha, K.*, et al.* Dyslipidaemia & oxidative stress in patients of psoriasis: Emerging cardiovascular risk factors. *Indian J Med Res* **146**, 708-713 (2017).
18. Rocha-Pereira, P.*, et al.* Dislipidemia and oxidative stress in mild and in severe psoriasis as a risk for cardiovascular disease. *Clinica Chimica Acta* **303**, 33-39 (2001).
19. Itani, S.*, et al.* High prevalence of metabolic syndrome in patients with psoriasis in Lebanon: a prospective study. *International Journal of Dermatology* **55**, 390-395 (2016).
20. Seishima, M.*, et al.* Serum lipid and apolipoprotein levels in patients with psoriasis. *British Journal of Dermatology* **130**, 738-742 (1994).
21. Gisondi, P.*, et al.* Psoriasis and the metabolic syndrome. *Clinics in Dermatology* **36**, 21-28 (2018).
22. Al-Mutairi, N.*, et al.* Comorbidities associated with psoriasis: An experience from the Middle East. *The Journal of Dermatology* **37**, 146-155 (2010).
23. Nisa, N. & Qazi, M. Prevalence of metabolic syndrome in patients with psoriasis. *Indian Journal of Dermatology, Venereology, and Leprology* **76**, 662-665 (2010).
24. Langan, S.M.*, et al.* Prevalence of Metabolic Syndrome in Patients with Psoriasis: A Population-Based Study in the United Kingdom. *Journal of Investigative Dermatology* **132**, 556-562 (2012).
25. Shiba, M.*, et al.* Risk of myocardial infarction in patients with psoriasis: A cross-sectional patient-population study in a Japanese hospital. *Journal of Cardiology* **73**, 276-279 (2019).
26. Organization, W.H. Definition and diagnosis of diabetes mellitus and intermediate hyperglycemia: report of a WHO/IDF consultation. (2006).
27. Gisondi, P.*, et al.* Prevalence of metabolic syndrome in patients with psoriasis: a hospital-based case–control study. *British Journal of Dermatology* **157**, 68-73 (2007).

CORRECT ANSWERS TO THE EXAM QUESTIONS

Answer question 1:
B. Fungal infections

Administration of the antibodies will lead to immunosuppression. The host's immune response against fungal infections is mostly regulated by neutrophilic granulocytes. These granulocytes are stimulated by the secretion of IL-17 by Th17-cells. Viral infections, on the other hand, are mediated by cytotoxic T-lymphocytes and natural killer cells.

For further reading:
Parham, P. Chapter 8: T-cel mediated immunity in The immune system, 4th edition. (Garland Science, New York, 2015)

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

Answer question 2:
B. Primary EBV-infection

After the presentation of an antigen, the B-lymphocytes first produce IgM, which forms the start of the immune response. During the ongoing infection, IgG is formed through isotype switching, resulting in long-term immunity.

For further reading:
Parham, P. Chapter 4: Antibody Structure and the Generation of B-Cell Diversity in The immune system, 4th edition. (Garland Science, New York, 2015)

During the exam, 60% of the participants answered this question correctly.

The exam questions can be found back on page 10 in this journal.

RECENT HIGH-IMPACT PAPERS FROM RADBOUDUMC RESEARCHERS

Yfke Prins[1]

Summary

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

[1] BSc Medicine, Radboud university medical center, Nijmegen, The Netherlands

Mutations in *TLR7* are associated with severe acute respiratory syndrome (SARS) in young male patients suffering from COVID-19

Severe acute respiratory syndrome is a life-threatening complication of the coronavirus disease 2019 (COVID-19) often requiring mechanical ventilation at the intensive care unit. When four males under 35 were admitted to the intensive care unit at Radboud and Maastricht University medical center, attention of the physician was caught, as the occurrence of this complication in young patients is rare and suggests a possible genetic component. Therefore, van der Made *et al*. analysed genetic variants in these four male patients that originated from two different families. The researchers performed whole-exome sequencing in the patients and compared the results with a control group. In all of the patients, a mutation in the X-chromosomal *Toll-like receptor 7* (*TLR7*) gene was found, albeit the mutations differed per family. In the first family, a four nucleotide hemizygous deletion, meaning that only one copy of the chromosome is present, was identified, leading to a total loss of function of TLR7 in both patients. Noteworthy, a heterozygous form of the deletion was observed in the mother. In the second family, a missense variant was found in *TLR7*. Furthermore, *TLR7* mRNA expression in peripheral blood mononuclear cells, derived from the patients, did not increase after stimulation with imiquimod, an agonist of TLR7. As TLR7 is involved in the generation of a type 1 interferon-γ response, these mutations will result in a diminished type 1 interferon-γ response. In addition, the fact that these mutations were detected on an X-chromosome bound gene could explain the observation that SARS is more frequent in male COVID-19 positive patients, as the male gender only has one X-chromosome. However, it is important to note that the detected hemizygous mutations are most likely quite rare. The question remains what the clinical effect of a heterozygous (carrier) mutation consists of, and if there are any genetic mutations associated with a mildly severe development of COVID-19. This research paper was published in the Journal of American Medical Association (impact factor 47.7) [2].

A phase I/IIa trial with a malaria vaccine using genetically attenuated *Plasmodium berghei*

Roestenberg *et al.* published a phase I/II clinical trial of a possible candidate vaccine against malaria in Science Translation Medicine (impact factor: 11.6). This is one of the first clinical trials using a live, genetically engineered strain of *Plasmodium berghei*, which is a malaria strain that causes malaria in rodents. A knock-out variation of this strain was created, lacking two proteins that are crucial for the development and growth of the sporozoites of *Plasmodium berghei* in the liver. This clinical trial consisted of phase I stage (n=19), assessing the safety of different dosages of the vaccination and a IIA stage (n=48) to establish the immunological effect and preliminary efficacy of the different doses. In order to assess the safety, the volunteers of the first group received three possible different doses of the vaccination or a placebo, with a follow-up lasting 100 days. The volunteers in the second group received the same varying dosages or placebo as the first group, after which a controlled human malaria infection (CHMI) was administered. The total follow-up in this group lasted 321 days. No serious side-effects were found in either group. The volunteers in the stage IIa-group receiving the lowest dose of the vaccination all developed parasitemia after CHMI. This pattern was seen as well for the volunteer group receiving the higher dosages, in which the majority also developed parasitemia. Nevertheless, 10% and 20% remained sterile, in the two groups receiving the highest dosages. All of the immunised volunteers showed an increase in antibody titers and an increase in interferon-γ-producing CD4+ and CD8+ T-cells, although only 35% and 32% of all volunteers were full responders in the production of CD4+and CD8+ T-cells, respectively. This clinical trial was one of the first to use a live genetically attenuated vaccine and proved that this could be administered safely, although further research is needed to truly establish and increase its efficacy [3].

Dolutegravir as a possible alternative to standard antiviral therapy in the third trimester of pregnancy in HIV positive women

The antiviral medication efavirenz is the current first-line treatment for human immunodeficiency virus (HIV) infection, although dolutegravir, an integrase inhibitor, has shown to lower the viral load in a significantly shorter time than efavirenez. However, the safety and efficacy of dolutegravir in pregnant, HIV-positive women, has not been evaluated properly. Therefore, Kintu *et al.*, part of an international consutim of scientists, published their clinical trial evaluating the use of dolutegravir as antiviral therapy in pregnancy in Lanclet HIV (impact factor: 14.8). A total of 268 pregnant HIV-positive women from Uganda and South-Africa, ranging from 29 to 34 weeks pregnant without prior treatment with antiviral therapy, were recruited. The volunteers received either dolutegravir or efavirenz, with a follow-up lasting until zero to fourteen days postpartum. With a median exposure time of 28 days needed to decrease viral loads to less than 50 copies per mL (95% confidence interval (CI) 28-34 days), dolutegravir was more efficient than efavirenz, which needed a median exposure time of 82 days to lower viral loads under 50 copies per mL (95% CI 55-97). The difference was smaller when looking at median time needed to decrease viral loads to less than 1000 copies per mL. The median exposure time needed was seven days in the dolutegravir group (95% CI 7-20) and 23 days in the efavirenz group (95% CI 21-27). More adverse effects occurred in the dolutegravir group in comparison to the efavirenz group, as 22% of women receiving dolutegravir and 14% of women receiving efavirenz developed at least one or more serious side-effect (p<0.013). However, in three volunteers treated with dolutegravir, *in utero* mother-to-child transmission of HIV occurred. Nonetheless, no significant difference was observed when looking at drug-related adverse effects. Therefore, in general, the medications were well tolerated. This research has provided the first step towards a possible new standard therapy for all patients with HIV, including HIV-positive women in late pregnancy [4].

References

1. Radboudumc. Jaardocument 2018. (2018).
2. Van der Made, CI., Simons, A., Schuurs-Hoeijmakers, J., Van den Heuvel, G., Mantere, T. et al. Presence of Genetic Variants Among Young Men With Severe COVID-19. *JAMA* **324**, 663-73 (2020).
3. Reuling, IJ., Mendes, AM., De Jong, GM., Fabra-García, A., Nunes-Cabaço, H. *et al.* An open-label phase 1/2a trial of a genetically modified rodent malaria parasite for immunization against Plasmodium falciparum malaria. *Science Translational Medicine* **12**, 2578 (2020).
4. Kintu, K., Malaba, TR., Nakibuka, J., Papamichael, C., Colbers, A., et al. Dolutegravir versus efavirenz in women starting HIV therapy in late pregnancy (DolPHIN-2): an open-label, randomised controlled trial. *The Lancet HIV* **7**, 332-9 (2020).

RAMS

A Word from the Board of RAMS

Dear reader,

Thank you for reading the seventeenth edition of RAMS. We hope that you have enjoyed this insight into the fascinating and comprehensive field of immunology, brought to you by our editorial board, editors, and other enthusiastic (bio)medical students involved. I would like to thank all of them for their contribution and dedication to our journal.

A new academic year has started, a new board has been seated, and a new edition has been published. Unfortunately, as much as we wished for this academic year to truly be a new beginning, back to our pre-pandemic lives, COVID-19 still remains among us. However, these troubling times also have some positive side effects; maybe you have finally had the time to learn a new skill, spent time with your family or bond with your roommates. Moreover, this crisis has thought us some valuable qualities; we have learned to be flexible, persistent and creative. All qualities that are crucial for you, as a future scientist or doctor.

The creativity that many of you have shown in order to stay connected to one another is something that we, as RAMS, also highly value this year. In addition to our journal, which connects us via our fascination for science, we are committed to bring you all together during our masterclasses, symposia and other new events of which you will hear soon. I hope to see you all there (virtually)!

On behalf of the board of RAMS,

Laura Hooijmaijers
Vice-chair of RAMS 2020-2021

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 Radboudumc. With their knowledge, the reviewers are able to judge the submitted scientific articles.

Privacy

RAMS conforms to the General Data Protection Regulation.
For our privacy policy see ramsresearch.nl/privacy-policy/

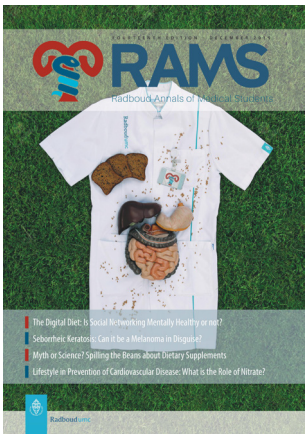
Do you want your article in the upcoming edition?*

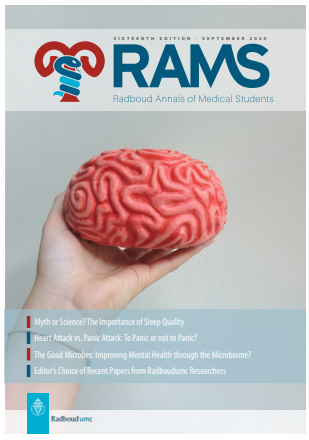
Send it to submit.rams@ru.nl or visit our website:

ramsresearch.nl

*Only with your supervisor's permission

Read our previous editions:





Radboudumc