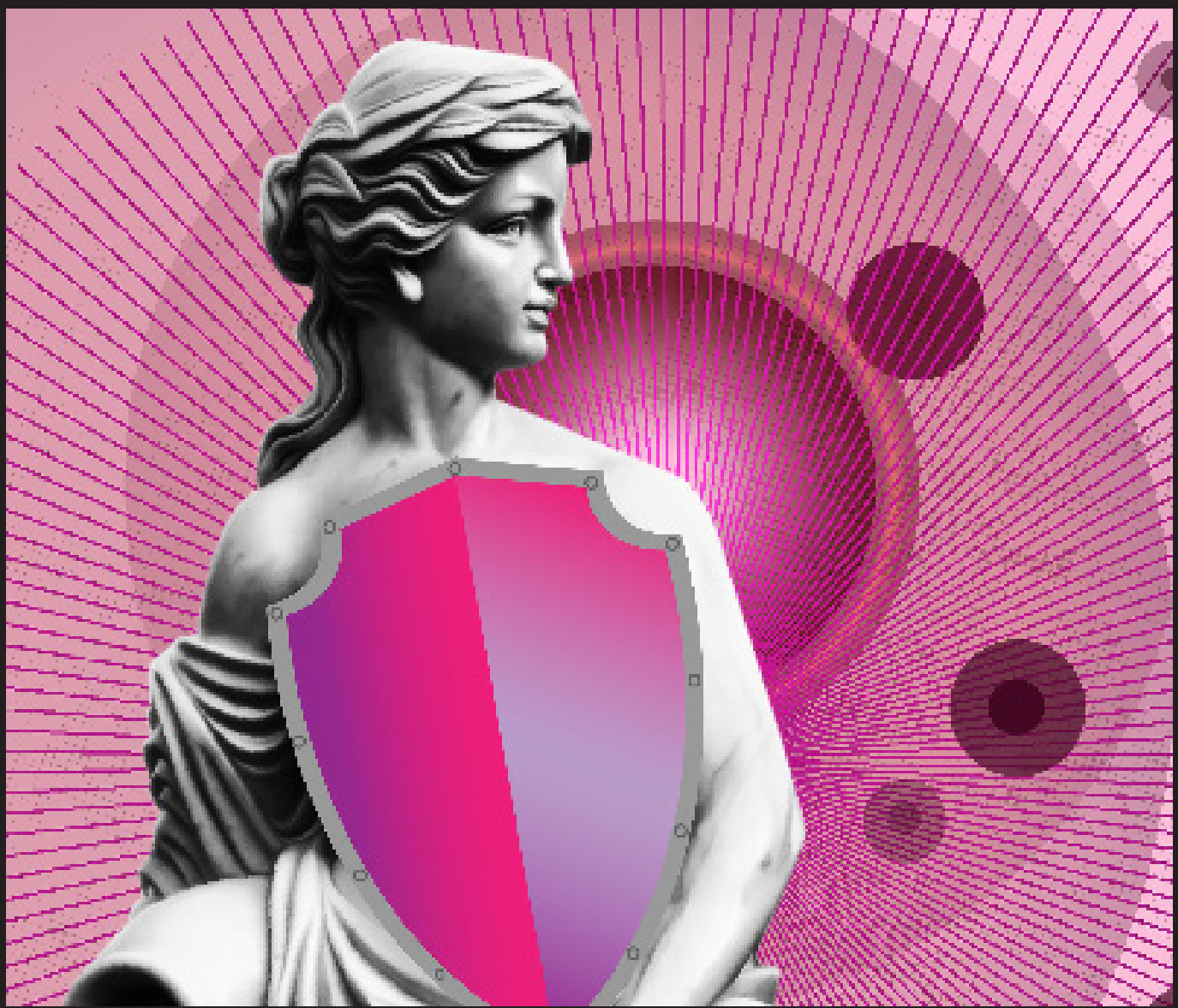
the Translational Scientist

MARCH 2017



Upfront

TREGeneration

Regulatory T cells can induce myelin regeneration

The complexity of the central nervous system (CNS) and our lack of understanding about its potential regenerative capabilities, mean that degenerative diseases such as multiple sclerosis (MS) – where the myelin sheaths of neurons degrade – have no cure. Pioneering researchers at Queen's University Belfast have been digging into demyelination and have revealed a new function for a subset of immune cells called regulatory T cells (Tregs) in the CNS: the ability to promote myelin regeneration (1). To learn more, we spoke with Denise Fitzgerald, lead investigator and senior lecturer at Queen's University Belfast.

What prompted you to investigate myelin regeneration?

Myelin regeneration (remyelination) can be a very efficient and effective process that restores function, but sometimes it fails and when it does, we have no therapeutics directed towards boosting remyelination – leaving a major unmet need for demyelinating conditions, such as MS. As an immunologist, I had researched how immune cells – particularly T cells – were involved in myelin damage, but there was very little information on how T cells influence myelin regeneration, so I felt that we could address some knowledge gaps. I thought that if we could identify how myelin is naturally regenerated, we could use that information to design potential new therapies to increase remyelination.

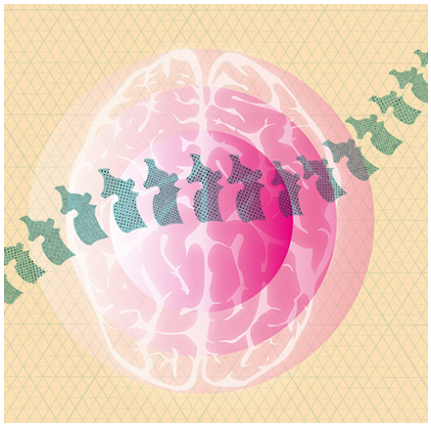
What do your findings mean for regenerative medicine?

Our results point to Tregs as key players in myelin regeneration. These cells have been identified as important in other regenerative sites – muscle and lung, for example – so our work increases the evidence that these cells play a role in tissue regeneration. What is striking, however, is that these cells may use different regenerative mechanisms in different tissues – it will be important to identify which aspects are tissue-specific mechanisms and mediators.

In our CNS studies, we identified the protein CCN3 as being a key product of Tregs that enhanced myelin production – which was surprising because i) the protein had not previously been known to be produced by T cells of any type, and ii) it was not known to be important in remyelination. In our experiments, we also found that neutralising/removing CCN3 was enough to abrogate the regenerative functions from Treg cells. As a T cell biologist, I expected that it would be a combination of factors or that compensatory mechanisms would be at play – and that may still be the case in more complex in vivo experimental models.

What were the main challenges?

As an immunology research group, we had to establish new techniques, which was only possible because we had fantastic support from our neuroscience collaborators. It was also incredibly challenging to design experiments that target Tregs in a way that modulates the regenerative phase of the experimental models, without solely reducing the extent of damage – in other words, reducing the initial myelin damage through immunomodulation is not the same as boosting regeneration. In the case of MS, we already have therapeutics that reduce damage with little impact



on myelin regeneration. Thus, we have to ensure that our preclinical studies are truly identifying changes in regeneration rather than changes in initial burdens of damage.

What's next?

We have several new projects that spawned as a result of this study, such as our translational work in human experimental models – examining interactions between different types of human Tregs and oligodendrocyte progenitor cells. We are also studying CCN3 in health and disease to determine its therapeutic development potential and its importance in general CNS function, as well as during de/remyelination. Another study is examining the molecular mechanisms of immune-mediated oligodendrocyte differentiation, the roles of other T cell subsets in myelin regeneration – as well as some slightly quirkier ideas that are at early stages so we can't quite discuss them yet... We are very grateful to the Wellcome Trust and BBSRC who are supporting our research programme.

Reference

1. *Y Dombrowski et al., "Regulatory T cells promote myelin regeneration in the central nervous system", Nat Neurosci, [Epub ahead of print] (2017). PMID: 28288125.*

A Good Axon Plan

Stabilization of protein–protein interactions could be the key to neuron repair

As the breadth of regenerative knowledge grows, so too does excitement surrounding discoveries with real potential – especially when the focus is on something as complex as damage to the central nervous system. Neurons typically have poor regenerative capacity, but investigators from McGill University (Montreal, Canada) have found a molecular interaction that appears to stimulate axon regeneration (1).

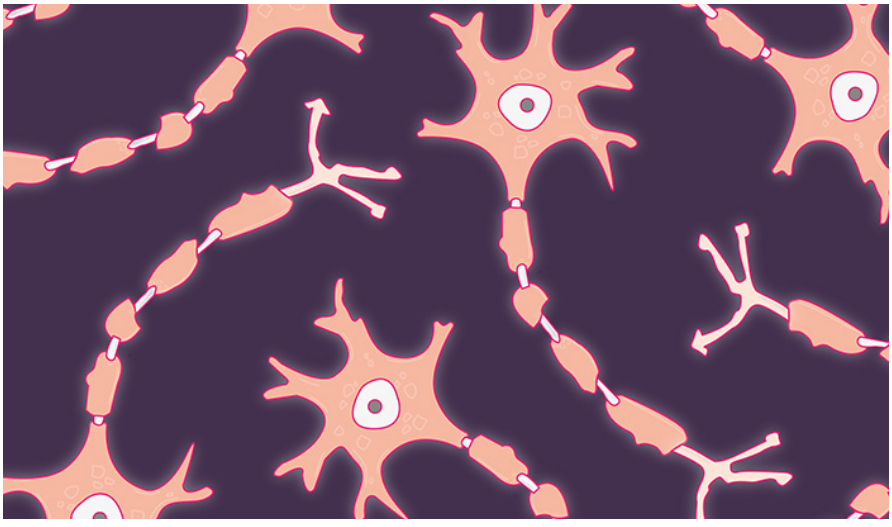
"Research from our lab – and from other groups – has shown that a family of proteins within neurons called 14-3-3s have beneficial functions in axon growth," says Andrew Kaplan, first author of the new paper. "We therefore searched for a pharmacological means to stimulate 14-3-3 interactions." What the team found was fusicoccin-A – a molecule that has the ability to tightly grip and stabilize 14-3-3 protein-protein complexes, which then stimulates in vitro axon growth and in vivo axon regeneration.

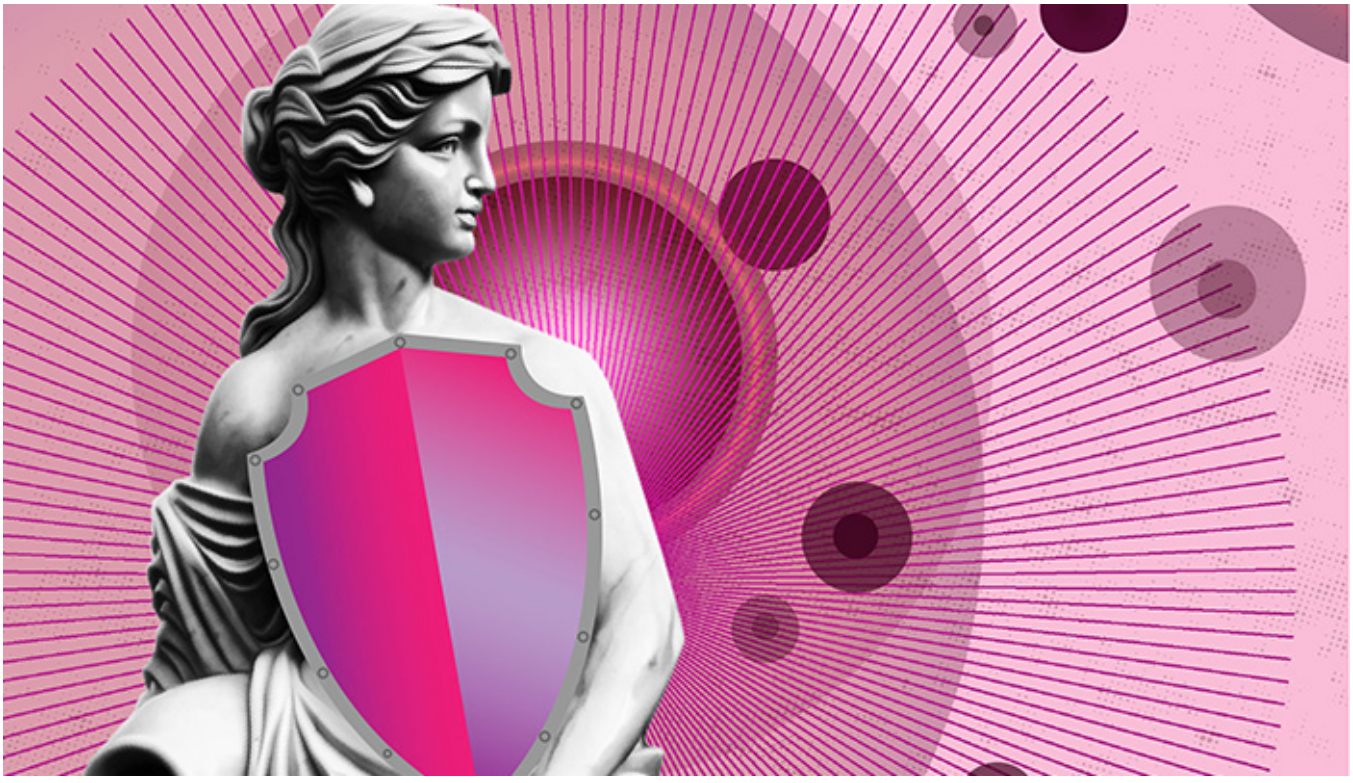
Could these findings be translated into the clinic? "Stabilizers of 14-3-3 protein-protein interactions, such as fusicoccin-A, may be able to direct the target proteins to stimulate repair of damaged axons after injuries. Kaplan says, "Our study shows that a repair mechanism can be unlocked by targeting the right factors within neurons. Essentially, nature has provided us with a key to unlock the potential of these proteins to stimulate axon repair."

With the key in hand, the next step is obvious: attempting to unlock the door to therapeutics. Kaplan says the team is currently pursuing a few lines of follow-up research with collaborators, including optimizing fusicoccin-A to improve its potency and activity. "We're also working on identifying the precise mechanism of action of fusicoccin-A, which could help us develop more targeted therapies to enhance axon repair."

Reference

1. *A Kaplan et al., "Small-molecule stabilization of 14-3-3 protein-protein interactions stimulates axon regeneration", Neuron, 93, 1082–1093 (2017). PMID: 28279353.*





Chemotherapy Plays a Role in Relapse?

Breast cancer stem cells may be surviving therapy... because of therapy

Breast cancer that resurfaces after initial successful treatment is often metastatic (1). Major players in that recurrence are breast cancer stem cells (BCSCs) – and a study by Johns Hopkins has found that chemotherapy is actually giving BCSCs a boost (2).

BCSCs sometimes evade the barrage of cytotoxic treatment by surrounding themselves with so many cancerous cells that the therapeutics can't reach them. Have the BCSCs simply traded in a deadly cytotoxic environment for a dangerous hypoxic one? No, because the crafty cells have another trick up their sleeve: the use of hypoxia inducible factor (HIF) proteins, which allow BCSCs to survive low-oxygen conditions.

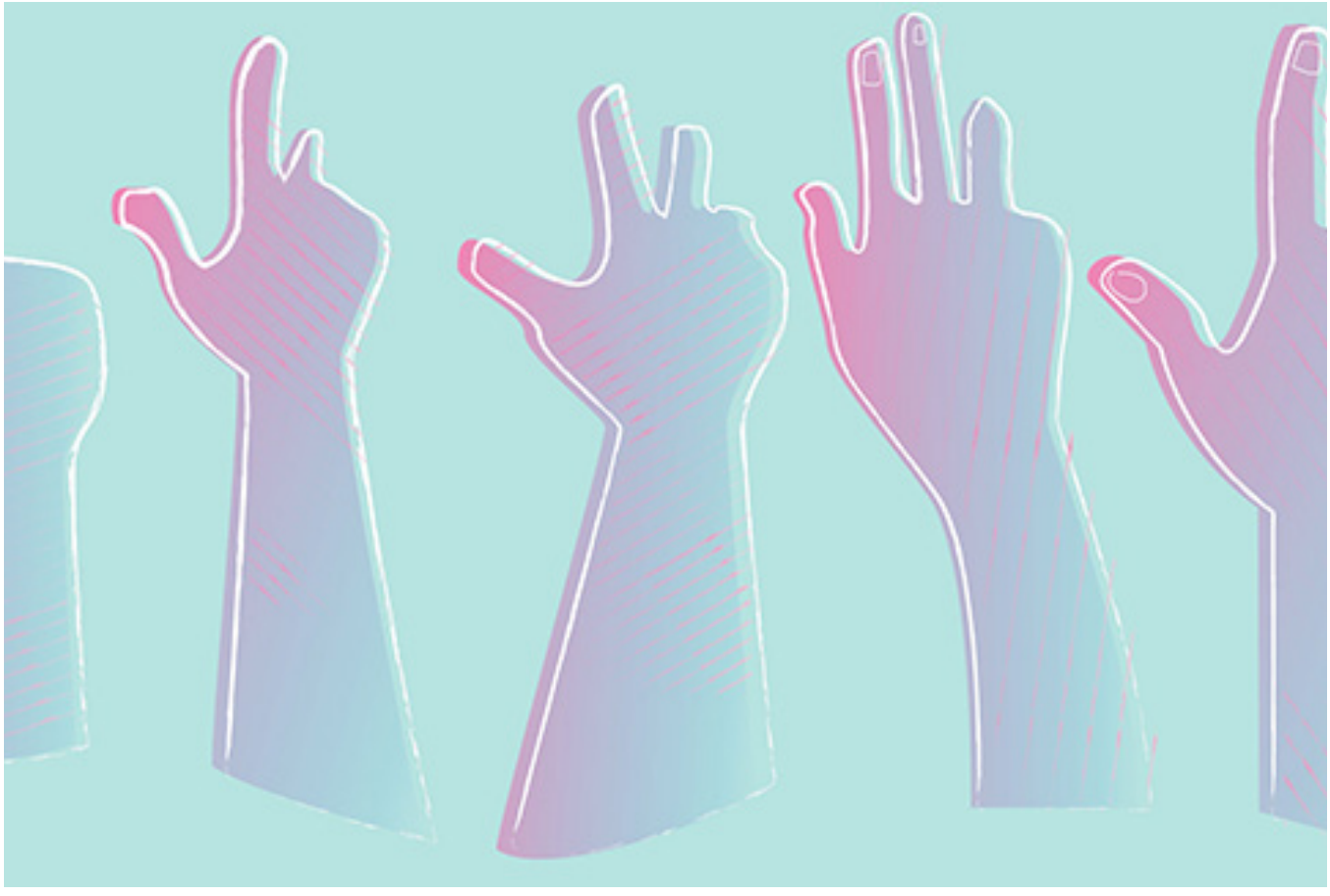
HIF determines the gene expression of GSTO1, which in turn promotes calcium release and leads to an increase of pluripotency factors and enrichment of BCSCs. The Johns Hopkins study found that some chemotherapies, such as carboplatin, can also induce GSTO1 expression and that HIF inhibition can reduce the effects of the chemotherapy-induced BCSC enrichment.

The results shine a light on an avenue by which the cancerous cells evade treatment – and could explain why cancer often comes back more aggressively after remission. The work also adds weight to previous claims (3) that HIF inhibition alongside chemotherapy could improve clinical outcomes and hints at another potential therapeutic target: GSTO1 inhibition.

The five-year survival rate after breast cancer diagnosis is around 90 percent (4) – counteracting the negative "side effects" of chemotherapy could push the number even higher.

Reference

1. *T Oskarsson et al., "Metastatic stem cells: sources, niches, and vital pathways", Cell Stem Cell, 14, 306–321 (2014). PMID: 24607405.*
2. *H Lu et al., "Chemotherapy-induced Ca2+ release stimulates breast cancer stem cell enrichment", Cell rep, 18, 1946–1957 (2017). PMID: 28228260.*
3. *D Samantha et al., "Hypoxia-inducible factors are required for chemotherapy resistance of breast cancer stem cells", Proc Natl Acad Sci USA, 111, E5429–E5438 (2014). PMID: 25453096.*
4. *National Cancer Institute, "Cancer stat facts: female breast cancer", (2017). Available at: bit.ly/2iGoIHA. Accessed March 20, 2017.*



Retinoic Regeneration

The RLR pathway gives us another immune clue in the search for regenerative growth

From starfish and salamanders to fictional clawed superheroes, regeneration is a trait that has fascinated many – not only because it's cool, but also because of its potential to revolutionize therapeutics. A team of researchers from Stanford University, Houston Methodist Research Institute, and Emory University School of Medicine, brought biomedicine slightly closer to realizing the dream, when they discovered another immune signaling pathway that could be manipulated to reprogram cells into pluripotency (1).

The investigators used loss- and gain-of-function studies to reveal that the retinoic acid-inducible gene 1-like receptor (RLR) signaling pathway is essential in the nuclear reprogramming of cells, which allows them transform into other organs or tissue. Could manipulation of the RLR pathway allow scientists to tap into regenerative properties by inducing the creation of pluripotent stem cells? Sounds like a positive next step.

The recent research adds to a growing body of knowledge in the field of regenerative medicine – the researchers previously showed the significance of Toll-like receptor 3 (TLR3) in nuclear programming (2) – deepening our understanding of the role that immune signaling pathways play in cellular manipulation. Though the ability to regrow whole limbs or eject bullets from gunshot wounds is some way down the road, we do appear to be one small step closer to a regenerative revolution...

Reference

1. *N Sayed et al., "Retinoic acid inducible gene 1 protein (RIG1)-like receptor pathway is required for efficient nuclear reprogramming", Stem Cells, [Epub ahead of print] (2017). PMID: 28204086.*
2. *J Lee et al., "Activation of innate immunity is required for efficient nuclear reprogramming", Cell, 151, 547 – 558 (2012). PMID: 23101625.*

Green Light for Pain Relief

LEDs are everywhere – and now they may be used to treat chronic pain

Despite chronic pain affecting approximately one third of the adult population in the US, the number of treatment options has remained relatively stagnant for decades. The Translational Scientist previously addressed the challenging nature of the search for new analgesics – and our experts urged other researchers to join the quest for novel therapeutic options (1). Now, a team at the University of Arizona has risen to the challenge and shown that light therapy could have uses beyond treating depression, jaundice, and skin conditions.

The researchers bathed rats under LED light at various wavelengths in the visible spectrum for 8 hours a day and compared their tolerance to neuropathic pain. Rats under 525 nm (green light) exhibited significant long-lasting antinociception (2).

To probe the mechanism, rats were fitted with opaque contact lenses and exposed to green light, or fitted with green contact lenses and exposed to room light; in the former, antinociception was prevented, but in the latter antinociception was exhibited, strongly indicating the role of the visual system.

More clinical work will be necessary to assess the potential of green LED therapy in the management of acute and chronic pain – but the lack of apparent side-effects, coupled with the low cost and wide availability of LEDs, sounds like a compelling treatment option for millions of sufferers worldwide. Perhaps equally importantly, the work shows that non-pharmacological approaches do exist and are worth seeking out...

Reference

1. *C Barker, "The Problem With Pain", The Translational Scientist, 3, 18–27 (2016). Available at: bit.ly/2lpYMN7.*
2. *MM Ibrahim et al., "Long-lasting antinociceptive effects of green light in acute and chronic pain in rats", Pain, 158, 347-360 (2017). PMID: 28092651.*

Pharmtastic Voyage

Scientists develop a microscopic "submarine" that neutralizes gastricacid for fuel to safely deliver drugs to the stomach

In the 1960's science fiction movie, Fantastic Voyage, Dr Jan Benes is left comatose with a life-threatening blood clot after a Soviet assassination attempt. Captain Bill Owens and his crew have only 60 minutes to board a miniaturized submarine and make their way to the clot, operate, and exit the body before they are returned to normal size, killing the unfortunate Benes.

Since its 1966 release, Fantastic Voyage has been widely celebrated, satirized, and now – with a new device developed by Jinxing Li and his team from the Department of Nanoengineering, University of California San Diego – imitated. Although there's no "atomic miniaturization" involved, Li's team have developed a microscopic "submarine" that can speed through the stomach using gastric acid for fuel (while simultaneously neutralizing it) and release cargo precisely at the desired pH. The "submarine" is actually a proton-driven, biocompatible micromotor with a pH-dependent polymer coating that can be loaded with drugs.

"Fantastic Voyage features a microscopic submarine that travels inside the human body to treat life-threatening issues," says Li. "We're aiming to apply our micro/nanomachines to drug delivery, detoxification and precision surgery."

A number of pH-sensitive compounds are vulnerable to gastric acid – including protein-based drugs and some antibiotics. An enteric coating can usually do the job of preventing degradation in the stomach, but for drugs that need to be activated in the stomach, for instance to treat stomach ulcers, proton pump inhibitors are usually needed to block gastric acid production. When used over longer periods, these can cause some side effects, including headaches, diarrhea, fatigue and, in some severe cases, rhabdomyolysis, a potentially life-threatening muscle disease.

Li's micromotors are microscopic spheres consisting of a magnesium core, which reacts with gastric acid to generate bubbles for propulsion. This process also neutralizes the stomach pH spontaneously (1). "Such motor-induced neutralization of the stomach fluid further triggers the autonomous payload release from the pH-sensitive polymer coating," says Li.

Where proton pump inhibitors suppress acid in the stomach, the micromotors alter the local environment without blocking the function of the proton pumps. "This approach hardly interferes with the function of the stomach and therefore completely eliminates the side effects associated with proton pump inhibitors," says Li.

Li hopes that the technology will help improve treatment efficiency since the propulsion of the micromotor allows it to penetrate the gastric mucosa, which increases the amount of time that the drug is retained in the stomach.

The journey continues as Li's team turn their attention to loading real therapeutic agents to treat stomach infections.

Reference

1. *J Li et al., "Micromotors Spontaneously Neutralize Gastric Acid for pH-Responsive Payload Release", Angewandte Chemie International Edition, (2017).*

Video of the Month

We previously highlighted the brilliant biomedical imagery of ex vivo lung perfusion (1) – circulating fluid through damaged lungs to stabilize them for subsequent use in a transplant. The main limitation of this earlier iteration was the approximate six-hour use of the apparatus, but a group of researchers from Columbia University have boosted that exponentially to 36-hour support of lungs.

The video shows an accelerated time-lapse of a pair of lungs being perfused for approximately 36 hours. In the top right is the patient data, while the bottom right displays the perfusion machine statistics.

View at: https://thetranslationalscientist.com/issues/mar17/video-of-the-month/

Credit: Gordana Vunjak-Novakovic/ Columbia Engineering.

Reference

1. *C Barker, W Aryitey, "The Art of Translation", The Translational Scientist, 7, 36 (2016). Available at: bit.ly/2mwjMSX.*

In My View

Change is Here, But Are We Ready for It?

Our approach to clinical research and translation must change if we are to deliver truly patient-centric healthcare

By Giorgio Stanta, Head of the Molecular Histopathology Laboratory, University of Trieste, Italy

The National Institutes of Health (NIH) defines clinical research as "Research with human subjects that is: Patient-oriented research. Research conducted with human subjects (or on material of human origin such as tissues, specimens, and cognitive phenomena) for which an investigator (or colleague) directly interacts with human subjects. Excluded from this definition are in vitro studies that utilize human tissues that cannot be linked to a living individual. It includes: (a) mechanisms of human disease, (b), therapeutic interventions, (c) clinical trials, or (d) development of new technologies" (1).

This seems clear and straightforward; however, as we continue to discover more about the genetic basis of disease and move ever more towards a personalized approach to diagnostics and therapeutics, the boundaries between what is defined as clinical research, and basic and translational research, are becoming blurred.

We have entered a new era in healthcare and medicine where patients' tests are being used to not only inform treatment decisions, but also to develop our knowledge of disease and drive new research. Recent advances in oncology – for example in molecular intratumor heterogeneity and its impact on the pathogenesis of disease and treatment resistance – have given rise to patient-centric clinical research performed on solid tissues or blood (the so-called liquid biopsies), with the results being very specific to that donor patient. In this scenario, molecular analyses are performed to verify clinical cases and to assess efficacy of new treatment opportunities. This analysis is not limited to a few defined biomarkers only, though, it gives rise to subgroups of patients, whereby some may have intrinsic resistance to therapy from the beginning, and others later present an acquired resistance. This type of knowledge supports the need for ongoing molecular analysis through a patient's treatment pathway with the definition of increasingly small groups of patients and suggestion of very specific combinatorial therapies. However, it also provides valuable information for the development of new therapeutics.

In anticipation of the growing importance of clinical research, the Organisation of European Cancer Institutes (OECI) (2) has developed a specific accreditation and designation programme for comprehensive cancer institutes. This accreditation takes into account not only the organization, diagnosis and therapeutic aspects of this molecular testing, but also what clinical research can be performed on what type of patient. The objective of the program is to guarantee that the patient has the most advanced treatment possibilities available, with a higher level of personalized analysis.

Accreditation is crucial for this type of research to ensure accuracy and reproducibility of results which, in my opinion, can be affected by at least three different factors. The first is the preclinical conditions of patients' material. For example, with fixed and paraffin-embedded tissues, long ischemia times

before fixation must be avoided, and sample acquisition and fixation should be performed with correct procedures in line with the recently developed CEN recommendations (3). The second aspect is the analytical methods used. These must be standardized and specific standard operating procedures should be followed, which include accurate internal and external quality control procedures. The third cause of irreproducible results is tissue and intratumor molecular heterogeneity, which is at the basis of clonal tumor evolution and acquired resistance to new therapies. This must be studied in depth, using tissues and "liquid biopsies" to define spatial and temporal development.

Understandably, this new approach to clinical research requires specialist facilities and is now viewed as an integrated activity in high-level clinical institutions. Not every hospital has direct access to these facilities though, and for this reason there is a real need for organized reference centres as an alternative for patients who need more sophisticated types of analysis and treatment.

Something else that needs to be very carefully considered is the bioethics of this type of approach. The fact is that we are using patient donor tissue or blood to support their own effective treatment, but also to perform clinical research, so this raises a number of bioethical issues. It's very important that this matter is discussed together with patient associations and an agreement reached on how to deal with it.

Overall, I strongly believe that new organizational changes are needed in health institutions. Clinical research must be central in this new vision, which must be developed together with patient organizations. Our new approach must support the training and continuous development of clinical researchers so that they amass experience and expertise in applying the results of clinical research to a single patient. In order to do this, however, we will need to create national and even international networks of reference centers, so that this level of patient-centric care can be made available to everyone, irrespective of their location.

Reference

1. *National Institutes of Health, "Glossary & Acronym List", (2013). Available at: bit.ly/2n39o9s. Accessed March 16, 2017.*
2. *OECI, "Working Group Accreditation and Designation". Available at: bit.ly/2kNEbXu. Accessed March 16, 2017.*
3. *CEN/TC 140, "In vitro diagnostic medical devices", (2017). Available at: bit.ly/2kmOxfG. Accessed March 16, 2017.*

How Serious Are You About Quality?

Fewer and fewer microbiologists are based in satellite laboratories, causing the accuracy of Gram stains to sometimes suffer. Telemicroscopy could be the solution to this worrying consequence...

By Linda Zuchowski, Midwest Regional Microbiology Manager, Quest Diagnostics Laboratories, USA

"Without a robust training and competency system in place to help support these generalists, Gram stain accuracy can be less then 90 percent relative to culture results."

Danish physician Hans Christian Gram first experimented with alkaline dyes to differentiate bacteria in 1884, and his staining method remains a valuable tool in microbiology labs today. Highly accurate, clinically relevant Gram stain results can quickly support or change an infectious disease diagnosis and lead to effective therapeutic decisions. But, physicians need confidence in Gram stain quality – "anything less than accurate, clinically relevant results is below the community standard of care" (1). Therefore, the lab must make every effort to provide high quality, timely Gram stain results to enable an acceptable standard of care for our patients. For some labs, telemicroscopy may be a key component of the solution.

In my view, providing highly accurate Gram stain results is not always easy. In our evolving healthcare climate, labs are consolidating and forming core microbiology labs, leaving many satellite lab locations without a microbiologist onsite. General technologists with little microbiology experience are then responsible for preparing and interpreting Gram stains in these satellite labs, which is far from ideal. Some struggle with

maintaining proficiency with this high complexity skill; they may have trouble making adequately stained slides, ending up with Gram-variable staining and vague results. Or, they may have difficulty identifying the bacterial morphology and thus hesitate to report the probable genus (2),(3),(4),(5).

Without a robust training and However, according to a poll at the 2015 annual American Society for Microbiology meeting in New Orleans, very few labs are actually using telemicroscopy. In my opinion, they are missing the many advantages of this progressive technology:

- easy and cost-effective consultation 24/7
- real-time slide review with microbiology experts across vast lab networks
- increased confidence and competency among less experienced technologists
- improved accuracy and patient outcomes
- confidential sharing of images using Windows IP configuration (no special software required)
- digital image library that can be used in training programs
- strengthened partnerships between core microbiology labs (or reference labs) and satellite labs
- application to any lab department using microscopy (hematology, parasitology, urinalysis)
- enhanced collaboration with public health officials during outbreaks and within bioterrorism preparedness programs

The cost of implementing a telemicroscopy system is minimal, especially when compared with the cost of revised reports or negative patient outcomes. With improvements in telemicroscopy and the advent of virtual Gram stain proficiency testing, this is absolutely the perfect time to incorporate digital technology into the microbiology lab. We all want to support the generalists in our satellite labs, share our expertise, and provide high quality, clinically relevant results for optimal patient outcome. Use of telemicroscopy to maximize the accuracy of Gram stain results is an effective way to achieve our mutual goals.

"Use of telemicroscopy to maximize the accuracy of Gram stain results is an effective way to achieve our mutual goals."

Reference

1. *EJ Baron et al., "A guide to utilization of the microbiology laboratory for diagnosis of infectious diseases: 2013 recommendations by the Infectious Diseases Society of America (IDSA) and the American Society for Microbiology (ASM)", Clin Infect Dis, 57, e22–e121 (2013). PMID: 23845951.*
2. *RL Sautter, RB Thomson Jr, "Consolidated clinical microbiology laboratories", J Clin Microbiol, 53, 1467–1472 (2015). PMID: 25253793.*
3. *J Barenfanger, CA Drake, "Interpretation of Gram stains for the nonmicrobiologist", Lab Med, 32, 368–375 (2001).*
4. *KH Rand, M Tillan, "Errors in interpretation of Gram stains from positive blood cultures", Am J Clin Pathol, 126, 686–690 (2006). PMID: 17050065.*
5. *DD Rhoads et al., "Clinical microbiology informatics", Clin Microbiol Rev, 27, 1025–1047 (2014).*

An End to Animal Testing is Not Yet in Sight

We need alternatives but, right now, in vivo methods might be the best option we have

By Bella Williams, Head of Engagement in Understanding Animal Research, and Sandy Mackay, Business Manager of Toxicology, Wickham Laboratories.

Let's consider the following scenario: new chemotherapy "Drug X" must be safety-tested before being given to volunteers as part of a clinical trial. Like all chemotherapy drugs, Drug X has been designed to be highly toxic and, if successful, will be an essential weapon in the cancer-fighting arsenal – reaching and killing cancer cells more rapidly than healthy cells.

As it will be used in immunocompromised patients, it is important that Drug X reaches the tumor without damaging the immune system

further, so safety tests include trials to discover its effects on the body's natural defenses.

Tests looking at immune reactions, along with a number of other essential toxicity tests for new drugs, have traditionally been carried out using animals, but more and more "alternative" non-animal assays are being developed to assess the toxicity of drugs and chemicals. These replacement tests are a regulatory directive – and research into alternatives is funded to the tune of 30 million euro through the Horizon 2020 program.

Although the development of new tests is a slow process that can take many years – especially as they must be validated through the European Centre for the Validation of Alternative Methods (ECVAM) – we have seen the successful rollout of non-animal replacements for standard toxicity tests. However, despite new methods being available, the organizations that carry out toxicity testing have been accused of slow adoption. Given that the majority of stakeholders want to reduce animal testing wherever possible, why do such organizations appear to drag their feet? The answer lies in the sector's responsibility to ensure that tests are carried out to the highest standards, which means balancing the best scientific results with the best achievable animal welfare.

Consider the rabbit pyrogen test, which assesses whether a particular compound elicits an immune response (the release of endogenous pyrogen from immune cells) and verifies the safety of a wide variety of products, including vaccines and other injectable drugs. Validated alternatives to this test have existed for some time; for example, the Monocyte Activation Test (MAT), which uses human cells to monitor the immune response. However, in some instances, human cells simply cannot process everything that a whole-body reaction in an animal can, so uptake of the test has been slow for companies working with complex compounds (for example, insoluble or cytotoxic substances).

Drug X is one such an example. As with many other chemotherapeutic drugs, it does not dissolve easily, so it must be encased in a lipid membrane that can be broken down by the body, allowing it to reach its target tumor cells. And because Drug X is cytotoxic, it damages the human cells used in MAT in any case. It is not possible to dilute Drug X and test the human immune response using in vitro cells, but a rabbit can break down the drug in a similar way to a human, which allows us to assess whether the drug may induce an unwanted immune response.

The specific example above may not be the only reason some companies may avoid MAT, which can be impractical or unsuitable for a wide variety of reasons. The test must be carried out multiple times to validate it against the target compound and ensure that an accurate reading is obtained, making it seem expensive and time-consuming (though this cost pales in comparison to the cost of maintaining a colony of experimental animals). The sensitivity of MAT has also raised concerns over the possibility of false positives, regardless of the fact that there are insufficient data available at this time to validate that concern. Finally, it only measures the release of pyrogen from cells; as the immune system is a complex system with many factors contributing to a possible immune response, there are also concerns that an in vitro method may not adequately reflect a whole-body system.

Other alternative tests have similar limitations. In vitro tests focus on the replacement of a specific organ, response or system, but are often unable to identify off-target effects identified through an immune system, metabolic or reflex response. Every compound being tested will have different requirements, and choosing the right test, whether animal or non-animal, will depend on understanding its interaction with the body.

In the future, we may even see previously used tests such as skin-sensitization tests making a comeback, if current alternatives are not sufficiently sensitive to provide appropriate data for a particular substance (for example, medical device leachables that may not be reliably detected using current in vitro methods).

Clearly, the work to develop more and better alternatives must continue – all stakeholders want to see fewer animals used in safety testing. And regulation demands that animals are only used where no alternative method exists, which means that where a validated method is available it must be used – but the validation of safety testing is not always so black-and-white.

Alternative tests need to be appropriate to both the compound and the scientific approach, providing data that meets or exceeds that gained from animal studies. Only by using a considered and scientific testing strategy can we ensure that the need to protect animals is balanced with the need to provide the highest quality data in safety testing.

Only Gene Deep

Advances in genomics are certainly thrilling, but let's not forget that a tumor is more than a bundle of genetic information.

By Han van Krieken, Chair of Pathology, Radboud University Medical Center, Nijmegen, Netherlands.

As histopathologists, we try to understand disease by looking at tissues. We see a snapshot of cells in their tissue environment. We can see whether they are normal or abnormal, whether there are too many or too few cells, how they are organized and how they interact. We can localize enzymes and proteins, measure expression levels, determine DNA alterations, and so on – and by bringing all this knowledge together, we can form a fairly complete picture of the disease that manifests itself in the tissue. These efforts provide the patient and the treating physician with information that can be used to choose the best possible treatment (or no treatment).

In this era of genetics, we are increasingly able to sequence the DNA of individuals and tumors, which allows us to quickly diagnose many different diseases that are caused by changes in genes, such as cystic fibrosis or Noonan syndrome. For cancers, we get information on the gene alterations that drive the tumor; for example, c-Erb2 amplification or ALK-fusions. Increasingly, it is suggested that whole genome sequencing will replace traditional forms of diagnosis. Indeed, if a child with an intellectual disability comes for a diagnosis, physical examination is already replaced by DNA analysis. And I was informed that in Hong Kong, where the incidence of EGFr mutated lung cancer is quite high compared with western countries, lung cancer is already diagnosed using genetic tests on blood samples in patients with inaccessible pulmonary lesions; if an EGFr mutation is found, it is regarded as sufficient evidence that the patient should be treated using an anti-EGFr approach. But in my view, although sequencing is an important diagnostic tool with much potential, it will never give the complete picture.

An example: it was recently shown that the cells within a tumor the size of a ping-pong ball will carry a total of 100 million mutations, with only a few of those mutations present in the majority of cells (1). Not only does this finding indicate that tumor heterogeneity on the cellular level is enormous, but also that complete sequencing of tumors provides us with so much data that it becomes useless. Quite interesting, of course, but not surprising for pathologists. In fact, that the nuclei in cancer cells are extremely variable compared to normal cells has been one of the most important criteria a pathologist uses when making a diagnosis of cancer for more than a century...

Furthermore, a tumor consists of not only neoplastic cells but also stromal cells, such as fibroblasts, inflammatory cells, endothelial cells and others. There is enormous variation in the ratios of these cell types between tumors – variation that has been shown to relate to treatment response and survival of the patient. Such variation cannot be found by sequencing the tumor or even the germline DNA.

Genes act through proteins, but proteins are not only modified by genetic mechanisms. Indeed, proteomic approaches are likely to give even more information, but replacing genomics with proteomics (which will take quite some time) will also not tell the whole story. Cells and tissues are so complex that we cannot fully understand what is going on by extracting only the genes and proteins. Spatial orientation, communication between cells, composition of tissues are all critical.

To that end, analyzing tissues with the microscope will remain an extremely cheap and fast way of providing useful information. But I am also convinced that we can benefit from new approaches in this field to extract even more information; for instance, deep-learning approaches – where standard tissue image analysis is supplemented with new information based on automated quantification of structures and protein levels – have great potential.

Of course, sequencing of tumors has given us a lot of valuable information – and will continue to do so – but we must remember that many other factors are equally important. As we all know, we are more than our genes – and a tumor is more than its genetic make-up.

Reference

1. *S Ling et al, "Extremely high genetic diversity in a single tumor points to prevalence of non-Darwinian cell evolution", Proc Natl Acad Sci USA. 112: E6496-505 (2015). DOI: 10.1073/pnas.1519556112. Erratum in: Proc Natl Acad Sci USA, 113: E663 (2016). PubMed PMID: 26561581*

How Are You – and How's Your Microbiome?

Exploration of the human body's 'second genome' could transform health and medicine – and robust analytical methodologies are key.

By Pratik Jagtap, Research Assistant Professor, Department of Biochemistry, Molecular Biology and Biophysics, University of Minnesota, Minneapolis, USA.

Microbes are everywhere. They are present in our gut, on our skin, in our oral cavity – and we may even carry a 'bacterial plume' around us. Our microbiota (the term used to describe the plethora of species present in an ecosystem) does more than just reside in our body, responding to changes within as we mature; recent studies have shown that the compositional balance of the microbiota and its expressed genes – the 'microbiome' – has a significant effect on health and well-being of the individual. Many pathological conditions, including allergies, inflammatory bowel disease, immunological disorders, type 2 diabetes, obesity, cardiovascular disease as well as mental health conditions, are influenced by the microbiome. Researchers describe the microbiome as the 'second genome' of the body – and there is potential to manipulate it to address disease states.

Both academic and clinical researchers are keenly studying the microbiome and its interaction with the environment, using techniques such as metagenomics, meta-transcriptomics and metaproteomics. Metagenomics – which studies the taxonomy of the microbiota (genera and species) helps in understanding the composition of microbiome. More importantly, meta-transcriptomic (RNA expression) and metaproteomic (protein expression) studies yield an insight into genes expressed by the microbiota as a community. It offers a much deeper understanding of how the microbiome interacts within the host environment – and affects the host. Though nucleic acid-based metagenomics and meta-transcriptomics are more sensitive technologies, metaproteomics offers insight into enzymes responsible for catalyzing reactions that affect the host. The metaproteome changes of the community (estimated by the functional categories of proteins expressed) offer a better indication as to how microbiota react to a change in the environment (for example, a disease) than estimation of the taxonomic composition of the community, which may remain unaffected.

The most interesting insights on how microbiomes affect our bodies in response to dietary habits have come from studies on malnourished or obese twins and on "germ-free" mice. When the gut microbiota of germ-free mice were replaced with microbiota from malnourished children and fed a poor diet, the mice lost weight and exhibited malnourished phenotypes (1). In another study, when the gut microbiota from obese person were introduced into the germ-free mice, they gained weight. And when the microbiota of obese mice were replaced with those from lean subjects, they maintained a normal weight if provided with a healthy, fat-reduced diet (2). On the other hand, studies on the effect of antibiotics on the human microbiome during the treatment of infections show that microbiota from even mature adults can change profoundly after antibiotic treatment (3). In addition to the increased threat of antibiotic-resistance caused by overuse, antibiotics can also have drastic side effects on the normal gut microflora.

To restore normal healthy flora, live microorganisms are administered in adequate amounts in 'probiotic' treatments. The biggest success story in the area of probiotics has been the use of fecal microbiota transplants (FMT), wherein fecal microbes from a healthy person are used to treat recurrent diarrhea caused by an antibiotic-resistant Clostridium difficile infection in a patient. FMT treatment is under investigation as a cure for other gastric disorders.

Although the results show a lot of promise, many microbiome researchers are taking a cautious and deliberate approach before suggesting cures to diseases. After all, researchers have only just started to explore the diverse microbiome and its complex interaction with the host. For example, Helicobacter pylori – a bacterium previously shown to be a causative agent in adults for digestive diseases (such as duodenal ulcer and gastric cancer) – has also been shown to have a protective role in esophageal premalignant and malignant conditions of the esophagus and also an inverse association with asthma and allergy (4).

Given the plasticity of our own genome and early successes in manipulating our 'second genome,' I believe the field of medical microbiology has the potential to deliver new therapeutic strategies not only for prevention of disease but also promotion of health. And analytical science must, of course, play an essential role.

Reference

1. *Smith et al, "Gut microbiomes of Malawian twin pairs discordant for Kwashiorkor," Science, 6119, 548-554 (2013).*
2. *Riduara et al, "Gut microbiota from twins discordant for obesity modulate metabolism in mice," Science, 6150 (2013).*
3. *Dethlefsen and Relman, "Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation," Proc. Natl. Acad. Sci. USA, 108, supp 1 (2011).*
4. *Backert and Blaser. "The role of CagA in the gastric biology of Helicobacter pylori," Cancer Res., 76 (2016).*

Feature

The Slow Tsunami Is Coming

And in its wake, a flood of drug-resistant superbugs... unless we do something to stem the tide

By Michael Schubert

Imagine that you're about to go on a holiday. You're packed and prepared, wavering between excitement for your upcoming trip and the familiar, slightly panicked feeling that you've definitely forgotten something. You're a little nervous about the flight, but as you take your seat, the pilot's reassuring voice comes over the intercom. "Good afternoon," he says. "This is a two-hour flight from London to Málaga. You have an 80 percent chance of safely reaching your destination." A one-in-five chance of disaster? Surely those odds are far too high for a simple vacation trip. But if that's the case, then why are such statistics acceptable in a healthcare setting? Dilip Nathwani, President of the British Society for Antimicrobial Chemotherapy, says, "We have a 50 to 70 percent chance of getting the right antibiotic for a patient's infection – and we sit and congratulate ourselves on those odds." To him, that's unacceptable – and that's only the surface of the problems with antimicrobial stewardship.

Antibiotic resistance is a critical issue in today's medical care, so much so that even the United Nations General Assembly felt the need to tackle it head-on. It's only the fourth time the UN has given such serious attention to a health issue, and they've taken a hard line, warning that antimicrobial resistance threatens worldwide development and requires a global response. All 193 member countries agreed – and all 193 of them have made a commitment to develop "superbug-fighting" action plans within the next two years.

What might these plans look like? There are three key aspects to a successful approach:

- Stewardship: a commitment to establishing and improving regulation and surveillance of antimicrobial use, sales and prescription;
- Research and development: not only of new types of antibiotics, but also of rapid diagnostics that can spot bacterial infections and identify effective treatments; and
- Education: both for healthcare professionals and for the general public.
- With drug-resistant infections estimated to claim 700,000 lives per year globally – and that number expected to grow to 10 million by 2050 (1) – it's not hard to understand why there's a need for immediate intervention. But the $64,000 (or, in this case, $100 trillion) question is: what can we do?

The slow tsunami

Antimicrobial resistance has been referred to as a "slow tsunami." There's a steady increase in the number of drug-resistant pathogen strains – and not just to hard-hitting drugs like the carbapenems, but also to the common, low-cost drugs that are often prescribed for minor ailments. Nathwani says, "I think that we're at a pivotal stage in the fight against antimicrobial resistance. One of the UN recommendations was for each country to have a plan in place within two years – but to me, the

challenge is much more than that. Not only do we need action plans, but we need to actually implement them and achieve some measurable goals." That, he says, is where the UN declaration falls short. "It doesn't really come up with targets. I think those are critical, because you need that kind of leverage within political and healthcare systems to bring about the change we all desire," (see "Steps to Success").

A lever and a place to stand

The problem – according to Elizabeth Tayler, Senior Technical Officer of Antimicrobial Resistance for the WHO – is that we as humans tend to engage in short-term risk avoidance, rather than taking a long-term view. "If there's a 5 percent chance that an infection might be bacterial, we'll treat just in case," she says. "If I take antibiotics, I might feel better slightly earlier, or be marginally less likely to get a secondary infection." It's a prevalent behavior, and one that can only be defeated through education. "The fundamental problem we have is making people more and more aware of the long-term risks."

To that end, the WHO teamed up in 2015 with the UN's Food and Agriculture Organization and the World Organization for Animal Health to develop a global action plan with five key objectives:

- **Education** "The first objective is to raise awareness among healthcare professionals, agricultural workers, and the public."
- **Surveillance** "The second is about strengthening our knowledge base around resistance patterns and consumption."
- **Infection prevention** "In low-income countries, a lot of that is about improving vaccination, water, and sanitation – community prevention. It's also important to improve infection control in health facilities and in the animal sector."
- **Stewardship** "We need to improve responsible use of antibiotics in both the human and animal sectors."
- **Resources** "We need adequate resources to do all of this, and we need to improve the models for drug and diagnostic development to ensure sustainable investment."

It's a plan that was endorsed by the UN General Assembly – "and so now," Tayler says, "what we have to do is action." The first step is well on its way. "I'm sitting in Trinidad at the moment, working with the Caribbean countries to develop their national action plans. To date, 32 countries have plans in place and 59 more are working on them. That includes the big countries – India, China, Brazil, Mexico and so on – who arguably have the most significant impact on antimicrobial resistance. Our biggest challenge now is to translate those plans into action, but we're seeing exciting signs that countries are beginning to take this seriously."

A global balancing act

Both Tayler and Nathwani are clear about one thing – that if we are going to succeed in defeating antimicrobial resistance, we need to take a broader view. This means considering not just the long term, but also the long distance. "If I take a global view," says Nathwani, "although we need to preserve the effectiveness of current antimicrobials, we also need to ensure that, in the parts of the world with little access to antibiotics, these treatments become available. There's a rather staggering statistic – that more people die from lack of access to antibiotics than die of drug-resistant infections. We need to reach a balance between stewarding our antibiotics and ensuring that everyone has sustainable access to them." It's a balance he hopes can be achieved by emphasizing infection prevention through methods like vaccination, hygiene, sanitation, and clean water – and by ensuring that, when antibiotics are made available, they're prescribed by professionals who understand their use.

Tayler points out that the rise in resistance to affordable antibiotics disproportionately affects resource-poor countries. "Those drugs have been the backbone of medicine in developing countries," she says, "but those countries are going to be very vulnerable if the drugs no longer work – so we're in a difficult position at the moment." And working within weak health systems to improve standards isn't easy. "Part of the problem is simple inertia. Even when people and organizations are enthusiastic for change, actually making it happen is another matter. "I think the impact is greatest in the poorest countries," says Tayler. "Those are the ones buffeted by Zika, by yellow fever, by political instability. It's interesting talking to people there, because there's a lot of enthusiasm – but when people change, or political systems change, inertia takes over."

Perhaps even more significant is the challenge of enforcement. "When you have poor or non-existent clinical governance, improving stewardship or enforcing regulations becomes much more difficult," says Tayler. "Although there can be quite good legislation, for instance as regards over-the-counter sales or restrictions on agricultural use, actually having the capacity to enforce that is very difficult – and there are plenty of people with a vested interest

Steps to Success

According to Dilip Nathwani, certain key measures must be taken to improve the chances of success of a program to tackle antibiotic resistance.

1. **Increase public understanding of the potential risks of antibiotics.**"They need to understand that the massive desire for antibiotics is counterproductive and harmful. These great therapeutic agents are also a threat, and abusing them can actually challenge and even negate many of the advances we've undertaken in medicine. That education needs to begin very early – from a school level."
2. **Increase healthcare professionals' understanding.**"We now have healthcare professionals outside medicine – nurses, pharmacists, dentists – who can prescribe, so we need them to engage with the principles of good prescribing. That's both an educational and a behavioral change.
3. **Adopt organizational empowerment.** "We need to make sure this happens in our communities, our nursing homes, and our hospitals. Here's a depressing fact: the United States has had extensive campaigns about antibiotic stewardship, but a recent study (2) showed that 55 percent of all hospital patients receive at least one antibiotic – and over the last decade, there has been no change in antibiotic consumption. So the question you have to ask is: what have all these attempted interventions actually achieved?"
4. **Take action – and track results.** "We need to see the measurable impact of all the good things we've been talking about for the last decade. Our focus must be on implementation, evaluation, and then further implementation – that is the proof in the pudding. Unless we do that, and unless we have systems to measure appropriateness and consumption and feed back to prescribers and the public, we won't bring about sustainable change."

in maintaining the status quo. It's a challenging environment in which to try to make progress." Nathwani chimes in with an example: "In India, where they have significant problems with unregulated antibiotic use, they've introduced a 'red line' concept. Antibiotic packages feature a dark red line, which signals that they should not be taken unless prescribed by a qualified professional. But that's very difficult to enforce, and its impact is as yet unmeasurable. So there's a lot of ambition, but actually enforcing the regulations is a huge challenge."

A question of resources

It's clear that developing countries will need special attention as we work to steward our antibiotics and stem the rise of resistance. But what about healthcare professionals in countries with more resources? The availability of funding and infrastructure doesn't guarantee that those things will be appropriately allocated – and there are already concerns that the approximately US$790 million pledged by the UN won't be enough (3).

"I think the resource question is critical. It's important that antimicrobial resistance is not seen as a specific project. It should be built in when we strengthen agricultural, health or laboratory systems – not treated as an add-on. Why? Because if we do it that way, it's much more likely to be sustained." Although we still have to make the case for additional resources, Tayler has some powerful arguments to suggest. "The O'Neill report (1) talked about a potential 2–3.5 percent fall in GDP by 2050. That's like something the size of the UK economy dropping out of the world market. The World Bank has done similar studies and says that the financial impact of antimicrobial resistance will be similar to the 2008 financial crisis, but much more protracted (4). These are the kinds of data that have a serious impact on policymakers."

That isn't to say that we haven't already made strides. "In the United Kingdom, we've seen fantastic results in primary care," says Nathwani. "We are beginning to reduce both overall antibiotic use and the misuse of broad-spectrum antibiotics." Nonetheless, he says, this impact – especially in hospital practice – needs to be greater still. In Scotland, where Nathwani practises, he says they've significantly reduced the use of cephalosporins and quinolones, but not total prescribing. "We must not be complacent," he warns. "We need to

focus our efforts on the hospital and long-term care facility setting – but without underestimating the importance of community prescribing, because 80 percent of all human prescribing occurs outside specialized care facilities."

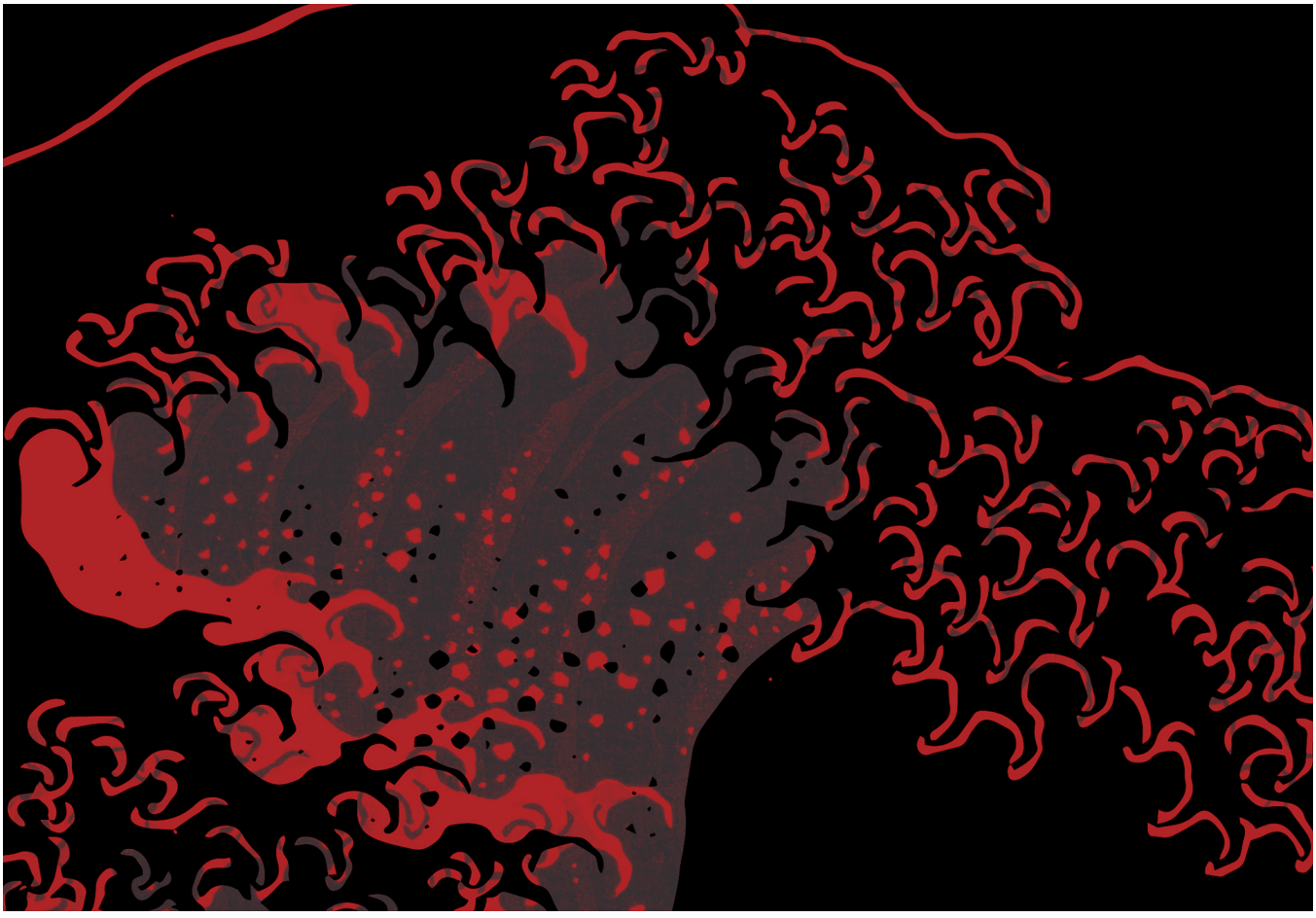
Maintaining momentum

So what's the biggest factor in all of this? Is it the amount of funding available? The pace of research into new drugs? According to Tayler and Nathwani, it's communication.

"It's quite interesting how you communicate things," observes Tayler. "Sometimes, people are much more captivated by the scary numbers; sometimes, it's the personal stories, or the fact that procedures like Caesarean sections, joint replacements or cancer treatment will become much riskier without the protective cover of antibiotics." She emphasizes, though, that the role of diagnostic professionals is critical. "You have the credibility, and also the local data. We are very short of good data in many contexts. At the WHO, we can talk about the global picture – but people are inherently worried about what's going on locally. Showing them that this problem exists in their own backyards focuses attention very well.

"People still trust doctors, and we need to leverage that to change the way we think about infection and antibiotics. We have a window of opportunity at the moment, while politicians are engaged and interested, but we know they'll move on. So it's vital that we put in a concerted effort to sustain that interest – or at least to revive it periodically."

"I think the clinical community needs to understand what the laboratory can offer," Nathwani adds, meaning not just the capabilities of medical science, but also its limitations. "Sometimes, I think the community expects too much of the laboratory. But if we understand each other and work together, then the outcome of each consultation will be more effective. I think pharmacists, nurses and infection specialists need to come together with primary care physicians to monitor antibiotic prescribing. Pharmacists can identify prescriptions that don't comply with policy; nurses can identify where intravenous drugs aren't required or question why a broad-spectrum antibiotic is in use where a narrower one would suffice. I think you



need a culture of effective teamwork across the disciplines, working very closely with the laboratory, to ensure that the quality of prescribing is good."

Nathwani's own hospital sets the standard. "We're quite protective of broad-spectrum antibiotics like the carbapenems. If one is prescribed on a surgical ward, the prescription will be reviewed by a pharmacist the next day – and if there isn't a good reason for it, that person will have a conversation with the attending team. If there's still no reasonable response, then the pharmacist will email the stewardship and infectious disease teams, and we'll review the patient that same day and make an analysis based on the clinical situation. And if we feel that the antibiotic is not appropriately prescribed, we'll have a conversation of our own with the attending team at a clinician-to-clinician level and recommend changing or stopping the drug. We also train and empower our nurses to get involved, asking questions like, 'Why can't we administer this treatment orally?' or 'Have you taken the blood level for this drug?' All of us – nurses, pharmacists, junior doctors, specialists – work together to optimize each prescription."

Damaging diagnostic discrepancies

"One major challenge to prescription reduction is diagnostic uncertainty. If you're a clinician and you're not sure of your diagnosis, then you're likely to prescribe or continue antibiotic treatment. I think that if we're really going to personalize medicine, we need a diagnostic test to determine whether infections are viral or bacterial – and, if bacterial, what antibiotics might be effective. We need these results in a timeframe rapid enough to inform clinical decision-making. And that's something we don't currently have." Nathwani believes that such tests hold the key to the future of antimicrobial stewardship – and he's not thinking only of those that are under development. Many biomarkers and point-of-care tests, like procalcitonin or C-reactive protein, already exist, but

simply aren't available in many areas. "Although we rely on new solutions," he says, "we already have effective tests that will reduce diagnostic uncertainty; they just need to be brought to the bedside more quickly. I think the laboratory community needs to embrace them and lobby for them to be made available – because these kinds of tests are cost-effective and will ultimately bring about a reduction in antibiotic consumption."

He does caution that new tests need to be combined with good stewardship, though. "Clinicians love sexy new diagnostics because they're 'better.' But their benefits are often overblown – and what they really do is make people over-investigate and overtreat. If you can't combine rapid diagnostics with specialists who are able to interpret them and provide advice, then the tests alone are useless."

Tayler's expectations for the future are similar. "I think that research on diagnostics is really important, because if we can get cheap, rapid tests that help people to prescribe more appropriately and manage the risk of missing something, that's a way to change behavior." She also believes that emerging evidence of the microbiome's positive effects will change people's perceptions of antibiotic risk. "If it's possible that I could be harming myself by taking antibiotics – not to mention the long-term harm to society – then I think that changes the inherent risk calculation, and I think that's important." She hopes that a desire to preserve the helpful microbiome may make patients think twice about demanding antibiotics when there's no real need.

Think big

Eric Reynolds, Melbourne Laureate Professor at the Melbourne Dental School, points out a major concern healthcare professionals may not always consider – the widespread use of not just antibiotics, but antimicrobials. "We have recently shown that long-term use of chlorhexidine (used as a disinfectant) is associated with the presence of multi-drug-resistant bacteria," he says. "The bacteria exchange genes for 'efflux pumps' to pump out small antimicrobial molecules like chlorhexidine and triclosan – but these efflux pumps can also be used to pump out conventional antibiotics. Therefore, these bacteria develop resistance not only to the antimicrobials, but also to antibiotics. They can then survive on skin or in mouths without causing any problems until we become otherwise immunocompromised." It's not just antibiotics that must be carefully stewarded, he warns – disinfectants and other antimicrobial agents need to be considered with the same level of caution.

"Clinicians love sexy new diagnostics because they're 'better.' But their benefits are often overblown – and what they really do is make people over-investigate and overtreat."

But how can we encourage such significant changes? Nathwani thinks that targets are the key. Measuring and feeding back data on consumption and quality is fundamental, because if that information is available to the clinicians and managers of healthcare systems, it can be used to inform metrics and future targets – and those can be powerful in influencing them to prescribe more effectively. "The other bit," he adds, "is to ensure that antibiotic prescription becomes a patient safety issue. I think that if you're prescribing poorly, then people coming into your healthcare facility should be told that they are not in a safe environment – and I think that kind of language will help people recognize that it's important to get this right."

Tayler has one more reminder for pathologists and laboratory medicine professionals involved in antimicrobial resistance. "I think this is one of the most exciting and important challenges that we face, so the work you're doing is massively important, and it's underpinning a global movement. Don't forget that – and instead of just focusing on the challenges that face your area, think about what you can do that will have the greatest global impact."

Reference

1. *The Review on Antimicrobial Resistance, "Antimicrobial resistance: tackling a crisis for the health and wealth of nations" (2014). Available at: bit.ly/1VOck4o. Accessed October 14, 2016.*
2. *J Baggs et al., "Estimating national trends in inpatient antibiotic use among US hospitals from 2006 to 2012", JAMA Intern Med, [Epub ahead of print] (2016). PMID: 27653796.*
3. *S Sekalala, "Superbugs 1, the world 0" (2016). Available at: bit.ly/2dVav7Q. Accessed October 14, 2016.*
4. *World Bank Group, "Drug-resistant infections: a threat to our economic future" (2016). Available at: bit.ly/2d8IQhS. Accessed October 14, 2016.*

Toolbox

At a Glance

- Cellular senescence can tell us a lot about tumor behavior but, until now, we've had no good way of detecting it
- Lipofuscin, a byproduct of lysosomal degradation, can identify senescence when detected by Sudan Black B staining
- Our new method capitalizes on this, but uses a much purer analog form of Sudan Black B than has been commercially available so far
- In the future, we hope to roll the new compound out to pathology labs worldwide – and expand to body fluid analysis as well as tissue staining

The Secrets of Senescence

On the back of an old technique – the histochemical detection of lipofuscin by Sudan Black B – we've built a new method for spotting cellular senescence.

By Vassilis Gorgoulis

Stemming from the Latin senex, meaning "to grow old," cellular senescence is a key stress response mechanism that preserves cellular homeostasis – which makes it important in normal physiology, embryonic development, and many pathological processes.

Let's imagine a cell in a stressful environment, being subjected to various insults. The cell has various ways of responding: it can die; it can enter arrest; or it can enter a state of senescence. In the latter state, the cell remains metabolically active, but doesn't proliferate. That's why we typically consider it an anti-tumor barrier – how can a cell be cancerous if it is incapable of replicating?

But there is also a dark side. A cell that remains in a state of senescence but isn't cleared from the organism eventually presents what is called the "senescence-associated secretory phenotype (SASP)." It releases cytokines that change the extracellular environment – and can transform the cell from a disease barrier into a disease promoter. How? Changes in expression of secreted factors can cause shedding of normally membrane-bound receptors, cleavage of signaling molecules, and even degradation of the extracellular matrix (1)(2). As a result, it's vital that we are able to detect senescent cells in clinical samples.

An enzymatic answer

Until now, the scientific community has only had one way of detecting senescent cells: the senescence-associated β-galactosidase assay, which measures the activity of the lysosomal enzyme β-galactosidase. Unfortunately, the method has more drawbacks than benefits. It can only be used in fresh tissue (not archival material), and its false-positive and false-negative rates are high. Knowing that we needed a better way to spot senescence, we turned to pathology's long history for an answer.

Lipofuscin (derived from the Greek word lipo, meaning fat, and the Latin fuscus, dark) is a byproduct of lysosomal digestion. A young Danish histologist, Adolf Hannover, first detected it in 1843 in the cytoplasm of nerve cells. Pathologists have been detecting these yellowish-brown granules ever since, but it never occurred to anyone that they could serve as indicators of a stressful condition like senescence. But here's the crux: when a cell is under stress, its bioenergetics can't keep up with demand, so lipofuscin begins to accumulate. We can then detect it using a traditional histochemical stain, Sudan Black B (3) – something that modern pathologists, who rely heavily on immunohistochemistry, may have overlooked.

Like senescence-associated β-galactosidase (SA-β-gal) itself, Sudan Black B has its pros and cons. Its key advantage is that it directly detects the cell's aging process via a waste product, rather than relying on enzyme levels. It also improves upon current false-positive and false-negative rates, allows multiple simultaneous stainings, and can identify senescence not only in cell cultures and frozen material, but also in archival material – a major step forward from the β-galactosidase assay. It's a technically challenging protocol, though; you need experienced pathologists to spot the Sudan Black B-stained lipofuscin granules, especially in the presence of background

"dirt." However, we believed we could remove that hurdle entirely by synthesizing our own highly pure Sudan Black B. We performed high-performance liquid chromatography on the commercially available dye, analyzed the spectrum of constituents and isolated the main component. Then we de novo synthesized its chemical analog and added biotin to it, generating GL13 – a new compound we can finally use in a sensitive and specific hybrid histo/immunochemical method.

Probing senescence mysteries

We are very proud of our discovery and its advantages over existing methods. I believe the scientific community will embrace it as the key method for the detection of senescent cells, especially as it can be expanded to other applications – immunofluorescence or flow cytometry, for instance. I'm also pleased that we've been able to provide something the field has needed for over 20 years: a tool for examining senescence in vivo. It's true that there have been biomarkers in the past, but none were specific; for instance, the tumor suppressor p16 has been used for senescence detection (4), but it also detects cell cycle arrest, so a p16-positive cell is not necessarily a senescent cell.

Essentially, we've added a third powerful tool to the evaluation of tumor kinetics; before, we could assess proliferation via Ki-67 and apoptosis via the apoptotic index, but now we have access to a third metric: senescence via lipofuscin. And if we see a tumor or other pathological entity with a high proportion of senescent cells, it means we have the opportunity to examine it further. Is it the "bright side" of senescence – the side that stalls tumor growth? Or is it the dark side that encourages the disease to progress? The answer to these questions lies in double stainings to detect SASP factors. If the staining is positive for SASP mediators, then tumor-promoting senescence features prevail.

When can we get hold of it?

As lipofuscin and Sudan Black B teach us more about senescence, I expect that many of the old questions will be answered and a lot of new ones will emerge. We're already seeing the new marker's inclusion in clinical trials, even though we only published less than a month ago (5). It seems the scientific community shares our enthusiasm! I've even had reviewers contact me to find out when our compound will be commercially available...

In answer to that question, I hope that the new method – and specifically, our highly pure GL13 – will become available to pathology departments in the next few months, because it's very important for the clinic. Until now, the only measure of a cancer patient's response to chemotherapy was the shrinkage of the tumor, which is caused by apoptosis. But what about the cancer cells that don't undergo that process, and instead enter senescence? The process makes them harmless but doesn't shrink the tumor. If we can include that parameter in our tumor kinetics, we can avoid giving patients chemotherapy they don't need. Better yet, we can now evaluate the effectiveness of novel therapeutic interventions that activate senescence and stall tumor growth. This is all-important: you can't kill something if you can't see it – a physician cannot choose an appropriate treatment unless he knows how the patient's disease behaves. Being able to measure senescent cells in tumors provides such an example by estimating how effective novel senescence-inducing therapies are. Moreover, this new method allows us to monitor the elimination of senescent cells in emerging rejuvenating therapies with senolytic drugs (6).

The future of senescence detection

Right now, we are on the verge of another major leap forward. So far, we've seen very positive results when testing the GL 13-mediated technique on samples of body fluids (for example, saliva and plasma), which is great because it will really boost the clinical applications. We can even combine the in situ tissue analysis with body fluid analysis for a more complete picture. And although this aspect is not yet fully developed, we believe that we will have it in the final stages as early as March!

As a final side note, I think the reason my colleagues and I were able to develop this new method is because we are also hybrids. I am a molecular pathologist – so I consider myself both a pathologist and a molecular biologist. There aren't that many of us in the world, but I think our ability to dive into both the basic and the clinical sides of research problems gives us added insight and helps lead us to advances like our new senescence test – and who knows how many others in the future?

Reference

1. *JP Coppé et al., "The senescence-associated secretory phenotype: the dark side of tumor suppression", Annu Rev Pathol, 5, 99–118 (2010). PMID: 20078217.*
2. *VG Gorgoulis, TD Halazonetis, "Oncogene-induced senescence: the bright and dark side of the response", Curr Opin Cell Biol, 22, 816–27 (2010). PMID: 20807678.*
3. *EA Georgakopoulou et al., "Specific lipofuscin staining as a novel biomarker to detect replicative and stress-induced senescence. A method applicable in cryo-preserved and archival tissues", Aging, 5, 37–50 (2013). PMID: 23449538.*
4. *H Rayess et al., "Cellular senescence and tumor suppressor gene p16", Int J Cancer, 130, 1715–1725 (2012). PMID: 22025288.*
5. *K Evangelou et al., "Robust, universal biomarker assay to detect senescent cells in biological specimens", Aging Cell, 16, 192–197 (2017). PMID: 28165661.*
6. *PL de Keizer, "The fountain of youth by targeting senescent cells?", Trends Mol Med, 23, 6-17 (2017). PMID: 28041565.*

Sitting Down With

Cell Therapy Virtuoso

Sitting Down With... Catherine Bollard, President of the International Society for Cellular Therapy (ISCT).

What inspired you to follow a medical career?

When I was 18 years old, I had to decide between medical school or opera singing. I chose medicine, thinking that I could revisit singing after qualifying – and I continued my voice training throughout medical school. After obtaining my medical degree, I moved to London to continue my opera training at Guildhall. But my husband was a musician too, and it was hard for a couple of poor musicians to live in London! I did a locum at St Bartholomew's in pediatric hematology and oncology – and I absolutely loved it. One day, when I was exhausted after being on call for 96 hours, my music teacher told me that I had to give up medicine. Medicine or singing? It was heart-wrenching, but I opted for medicine. I still keep up the music as a hobby – and I think the characteristics of a good doctor are similar to those of a good musician: one fixes the body and the other mends the soul.

How did you become interested in cell therapies?

In the 80s, my best friend in high school, Diana, developed Hodgkin's lymphoma. Treatment comprised multiple cycles of chemo- and radiotherapy, but finally she went into remission. Later, she was diagnosed with myelodysplastic syndrome – a direct consequence of the Hodgkin's therapy – and died soon afterwards of leukemia. It was so cruel, and it became clear to me that we needed therapies that only kill the cancer cells and not healthy bystander cells. As a result, I became interested in cellular immunotherapy.

My mentor in New Zealand was friends with Helen Heslop and Malcolm Brenner – two leading immunotherapists at Baylor College of Medicine in Houston, USA – and I agreed to work with them on neuroblastoma. But when I arrived, the neuroblastoma post-doc decided to stay on, while another (who had been working on Hodgkin's lymphoma) left, so I was able to work on lymphoma – one large focus of my career ever since. We've now developed a T-cell therapy that gives complete remissions in over 50 percent of some patient groups, and two-year progression-free survival rates of over 90 percent in other groups. Cell Medica is taking this product to licensure. It's exciting, but also very sad. If Diana had been diagnosed today, her outcome would have been completely different.

How did you get involved with ISCT?

When I was at Baylor, my boss, Dr Brenner, was President of ISCT so of course he encouraged us all to join! I really enjoyed its bench-to-bedside philosophy and the fact that it encompassed all aspects of cell therapy. I became President in 2016.

What is ISCT's current focus?

ISCT's mission is to "drive the translation of cellular therapies for the benefit of patients worldwide" and to "improve patients' lives through safe and effective cellular therapies". At the moment, we're very focused on

maximizing our growth in terms of membership, as well as reaching out to the community through current and new collaborations. As new cell products become available, I would like to see ISCT support the IND-enabling studies that bridge "the valley of death" – the early phase studies where success is critical for generating corporate interest and later phase licensing studies.

What are your goals for 2017 as President of ISCT?

In recent years, the society has enhanced its regenerative medicine profile, and I now wish to build its profile in immune cell-based therapies, especially in cancer immunotherapy. 2017 marks ISCT's 25th anniversary and I'm hoping to engage past-ISCT presidents – who were the early drivers of cancer cell therapy – to re-energize this arm of ISCT.

The ISCT also has a responsibility to help younger professionals develop the necessary skills to contribute to the field. Training the next generation of cell therapists is a passion of mine; in 2015, I helped develop an intensive cell therapy training course with the American Society of Blood and Marrow Transplantation. The first course was very successful so we're doing another this year.

What do you think will help to further advance the field?

I'd like to see more collaboration between pharma companies – not only in terms of developing a given cell therapy, but in the development of combination treatment strategies utilizing immune and cell based therapeutics. For example, if cancer is to be cured, it will most likely require immunotherapy combinations, which will require this sort of collaboration. ISCT is well-positioned to facilitate collaboration between pharma companies as our commercialization committee has developed close working relationships with industry and academia.

What are the most exciting changes you've seen in the cell therapy field?

The field has expanded dramatically over the last 25 years. In particular, T-cell therapies for cancer have grown rapidly and now the field is expanding into other areas, such as regulatory T-cells for autoimmune disease and virus T-cells for HIV. Given what the immune system can do, the applications are almost limitless. Technological advances now place this diverse array of treatments within reach of generalizability. Ongoing issues include the question of whether we should have centralized or decentralized GMP facilities – or perhaps even a "GMP in a box" approach where blood and reagents go in and your product comes out!

ISCT will celebrate its silver jubilee at its upcoming annual meeting on May 3-6 in London, UK. Visit http://isct2017.com/ for more details.

Imprint

STAFF:

Editor - Charlotte Barker
charlotte.barker@texerepublishing.com

Associate Editor - William Aryitey
william.aryitey@texerepublishing.com

Editorial Director - Fedra Pavlou
fedra.pavlou@texerepublishing.com

Content Director - Rich Whitworth
rich.whitworth@texerepublishing.com

Publisher - Mark Goodrich
mark.goodrich@texerepublishing.com

Head of Design - Marc Bird
marc.bird@texerepublishing.com

Designer - Emily Strefford-Johnson
emily.johnson@texerepublishing.com

Junior Designer - Hannah Ennis
hannah.ennis@texerepublishing.com

Digital Team Lead - David Roberts
david.roberts@texerepublishing.com

Digital Producer Web/Email - Peter Bartley
peter.bartley@texerepublishing.com

Digital Producer Web/App - Abygail Bradley
abygail.bradley@texerepublishing.com

Digital Content Assistant - Lauren Torr
lauren.torr@texerepublishing.com

Audience Insight Manager - Tracey Nicholls
tracey.nicholls@texerepublishing.com

Traffic and Audience Associate - Lindsey Vickers
lindsey.vickers@texerepublishing.com

Traffic and Audience Associate - Jody Fryett
jody.fryett@texerepublishing.com

Apprentice, Social Media / Analytics - Ben Holah
ben.holah@texerepublishing.com

Events and Office Administrator - Alice Daniels-Wright
alice.danielswright@texerepublishing.com

Financial Controller - Phil Dale
phil.dale@texerepublishing.com

Chief Executive Officer - Andy Davies
andy.davies@texerepublishing.com

Chief Operating Officer - Tracey Peers
tracey.peers@texerepublishing.com

PUBLISHED BY:

tracey.nicholls@texerepublishing.com
Tracey Nicholls, The Translational Scientist, Texere Publishing Ltd, Haig House, Haig Road, Knutsford, Cheshire, WA16 8DX, UK

GENERAL ENQUIRIES:

info@texerepublishing.com

+44 (0) 1565 745200

sales@texerepublishing.com

DISTRIBUTION:

The Translational Scientist (ISSN 2397-0588),
is published by Texere Publishing Ltd and is distributed in the USA by UKP Worldwide, 1637 Stelton Road B2, Piscataway, NJ 08854.

Periodicals Postage Paid at Piscataway, NJ and additional mailing offices

POSTMASTER: Send US address changes to The Translational Scientist, Texere Publishing Ltd, C/o 1637 Stelton Road B2, Piscataway NJ 08854

Single copy sales £15 *(plus postage, cost available on request tracey.nicholls@texerepublishing.com)*

Annual subscription for non-qualified recipients £110

Reprints & Permissions – *tracey.nicholls@texerepublishing.com*