APRIL 2016 # 18

the Pathologist

Cells on the (Invasion) Front Lines

New research asks: do tumor cells really need EMT to metastasize?

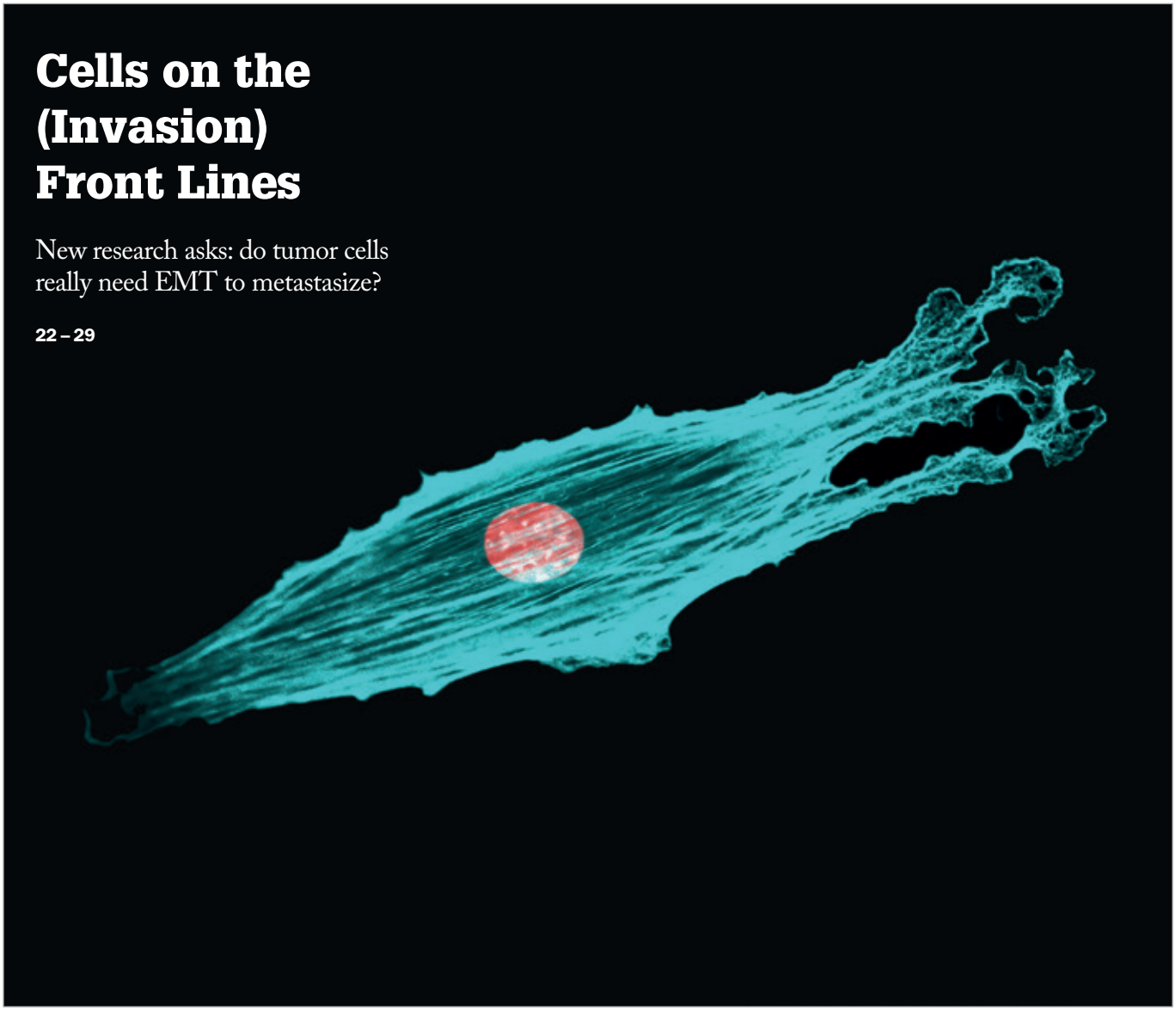

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Image of the Month

This image of gallstones, at 4x magnification, was supplied by Norm Barker, Professor of Pathology & Art as Applied to Medicine and Director, Pathology Photography, Digital Imaging and Computer Graphics Laboratory at The Johns Hopkins School of Medicine. The variability in color and texture of these solid crystalline deposits arises from differences in the stones' primary compounds. Gallstones were known as early as 2000 BC, when they were mentioned in divination texts that claimed they could represent either a good or a bad omen. Today, however, you would be hard-pressed to find a gallstone sufferer holding up their disease as auspicious!

Do you have an image you'd like to see featured in The Pathologist?
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On The Cover

Do tumor cells – like the metastatic cell depicted on this issue's cover – really need the epithelial-to-mesenchymal transition to invade?

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the Pathologist

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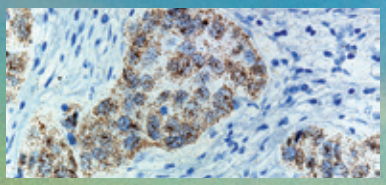

HER2 expression in human breast cancer FFPE tissue using RNAscope® 2.0 HD Reagent Kit-BROWN

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A Question of Value

Molecular testing is changing the face of healthcare. But how far should it go?

You can't attend an oncology conference or read a medical journal (or even an issue of The Pathologist) these days without hearing about the latest genomic discoveries. In fact, even as I write this editorial, my inbox is filling with interesting announcements. One that particularly caught my attention was a paper authored by an international group, calling for the reclassification of a thyroid tumor (1). Encapsulated follicular variant of papillary thyroid carcinoma (EFVPTC) is currently treated as a conventional thyroid cancer, but according to the group, it carries a very low risk of adverse outcome. After combining histologic and molecular analysis of EFVPTC tumors, the researchers make a strong recommendation: relabel the tumor so that its name more accurately reflects its nature and, more importantly, doesn't contain the dreaded "C" word. The impact could be a profound one: remove the psychological stress of a cancer diagnosis for thousands; improve treatment outcomes; save money lost to overtreatment…

There's little doubt that our growing knowledge of disease subtypes and the move to genetic testing are having an overall positive impact on patients' lives. But a question recently posed to the clinical community has yielded strong, differing opinions: should universal tumor sequencing be available for all cancer patients? I read some very interesting reflections from people in both camps.

Authors from the Moores Cancer Center at the University of California San Diego remind us of the staggering financial burden of cancer in comparison to the cost of genomic testing (2). They also refer to comprehensive studies that demonstrated improved outcomes in patients given personalized therapy and highlight the high value of the data collected. Their recommendation? Pathology-driven reflexive testing to ensure that all tumors are evaluated, because "if we are serious about winning the war against cancer, we should gather every bit of intelligence about it. A half-hearted approach to any battle would only lead to failure."

In stark contrast, Howard West of Seattle's Swedish Cancer Institute likened proponents of universal sequencing to patients enthused by the remarkable therapeutic benefits of alternative treatments like cannabis oil or vitamin C infusions (3). West claims strongly that there is insufficient evidence to support universal testing, in particular because of the lack of data on how many patients undergo molecular testing beyond current standards, and refers to studies that failed to demonstrate any improvement in progression-free or overall survival. "We cannot realistically anticipate that this effort will broadly improve cancer outcomes any more than we could expect to eradicate poverty by distributing lottery tickets to the poor," he boldly states.

It will certainly be interesting to see how this one plays out…

Fedra Pavlou
Editor

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Michal Michal

As head of Bioptická Laboratoř in Plzeň, Czech Republic, master organizer and first-class pathologist Michal Michal has cornered the majority of private pathology business in the country. His laboratory continues to expand, buying and transforming real estate and moving into neighboring countries like Slovakia, where they control nearly one-third of pathology business. He and his group are active in presenting, publishing, and participating in national and international meetings – and with over 600 peer-reviewed papers, Michal himself continues to be a driving force for pathology in Eastern Europe.

Turn to page 46 to read the inside story of Bioptická Laboratoř.

Shyamala Maheswaran

Shyamala, a veteran of both academia and biotechnology, is currently Associate Professor of Surgery at Harvard Medical School and Assistant Molecular Biologist at the Center for Cancer Research, Massachusetts General Hospital Research Institute, Boston. She runs a research group focused on understanding the biology of tumorigenesis and metastasis, including investigating the roles of the epithelial-to-mesenchymal transition, examining the tumor microenvironment, and profiling the molecular characteristics of circulating tumor cells (a multidisciplinary project she co-directs with Daniel Haber and Mehmet Toner).

Shyamala shares her views on the role of EMT in metastasis on page 24.

Susan Rollins

Susan is Associate Professor of Pathology and Medical Director at the Outpatient Cytopathology Center of East Tennessee State University, Johnson City, USA. Shortly after moving to Tennessee, she opened an independent fine needle aspiration (FNA) biopsy clinic in Johnson City. When her husband, an interventional radiologist, changed his career path, Susan was left without help for patients needing ultrasound-guided (US) FNA biopsies. So she mastered the technique herself – and now, in addition to a robust USFNA practice, she teaches the technique to pathologists and other physicians in training.

Read Susan's opinions on the value of fine needle aspiration on page 20.

Laura Lechuga

A Full Professor at the Spanish National Research Council (CSIC), Laura is also head of the Nanobiosensors and Bioanalytical Applications Group in the Catalan Institute for Nanoscience and Nanotechnology. "My main focus is the technological development of photonic (plasmonics and silicon-based) and nanomechanical biosensors, their integration in portable lab-on-a-chip platforms, and their application in clinical and environmental diagnostics. It is a truly fascinating area of research that will open up new opportunities in point-of-care diagnostics and will help take healthcare directly to those in need," she says.

Laura talks about the potential of point-of-care smartphone testing on page 19.

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Under the Skin

A blood test for circulating tumor DNA can reveal what's going on below the surface of malignant melanoma

Even the first time around, melanoma is a frightening diagnosis. But for patients who experience a recurrence or metastasis, the outlook is much worse and the options far fewer. In cases like these, the sooner the relapse is discovered, the better the chance of treating the disease and extending patients' survival times. To that end, scientists from the Cancer Research UK Manchester Institute have developed a blood test that may not only detect the first signs of returning cancer, but also yield information on mutations that can lead to treatment resistance.

The study examined blood samples from seven patients with advanced melanoma, tracking both levels of circulating tumor DNA (ctDNA) and the mutations present (1). Richard Marais, senior author and Cancer Research UK's skin cancer expert, explains, "We monitor levels of mutated DNA in the blood of melanoma patients using an assay that allows us to simultaneously trace 10 genomic loci associated with resistance to targeted therapy. When following patients on *BRAF*-targeted therapy, we look for increasing levels of the *BRAF* mutation that the patient was originally diagnosed with, and at the same time, we try to pick up new mutations that may be the cause of resistance."

The analysis of ctDNA is thought to offer a number of advantages over tissue biopsies: convenience to the patient, cost-effectiveness to the healthcare provider, and the detection of mutations from multiple metastatic sites while avoiding the selection bias associated with tissue biopsies. "Overall," says Marais, "the analysis of ctDNA represents an ideal tool for the implementation of personalized medicine, and to complement standard imaging procedures to monitor disease progression."

What might this research mean for the clinic? Marais anticipates the development of molecular profiling kits for ctDNA analysis to complement tissue profiling. "The analysis could easily be performed by pathologists," he says, "and the results interpreted by the multidisciplinary teams treating the patient." He and his colleagues hope that the analysis of ctDNA will help tailor the clinical management of patients to their needs, supplementing current disease-monitoring practices. "Blood can be collected on a regular basis at clinic visits by a local general practitioner, or even in the comfort of a patient's home. Fixed intervals between scans could be replaced by a flexible schedule, giving priority to patients showing early signs of disease progression in the blood, or even delaying scans for patients whose blood indicates a good response to treatment." He emphasizes that the technique would not replace current approaches, but rather would be used to refine patient care.

At the moment, the approach is ideal for the longitudinal follow-up of patients with pre-described mutations, but the team are aiming to determine the capability and applicability of their test for patients at risk of developing stage IV melanoma. In the meantime, they're busy establishing ctDNA analysis as a clinical tool, testing the application of different technologies to prospectively monitor a larger cohort of patients, and incorporating their protocols into new clinical trials.. *MS*

Reference

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Power to the People

Direct-to-consumer lab tests offer low cost and convenience, but can the results they return be relied upon?

Opinion on direct-to-consumer (DTC) lab tests is divided – while some believe that patients should take charge of their own health in every way, others are rightfully concerned that untrained eyes might misunderstand the meaning, significance or reliability of the results. Regardless of expert opinions on the subject, as medical technology becomes increasingly accessible, it seems that consumer testing is here to stay. But what is it that patients are learning from these tests – and can it be trusted? To find out, a team of researchers from Mount Sinai's Icahn School of Medicine embarked on a cohort study of healthy adults using popular over-the-counter tests (1).

To examine the performance of these tests, 60 adults were administered 22 common laboratory tests from either a DTC company (Theranos, which uses fingerprick diagnostics) or clinical testing services (Quest and LabCorp, both of which use venipuncture). The tests included a complete blood count panel, leukocyte subsets, lipid panel, inflammation, kidney and liver labs. The blood draws were completed within the span of 6.5 hours; each patient had one venipuncture and one fingerprick early in the day, then another of each draw late in the day.

What did they find? The DTC test returned missing data more often than the clinical services (2.2 percent, as compared with 0.2 and 0 percent) and reported more measures outside the normal range (12.2 percent, as compared with 8.3 and 7.5 percent). Most notably, results for mean corpuscular hemoglobin concentration, lymphocyte count, and cholesterol components were out of range more often than expected, with ratios between the consumer test and the lab services ranging from 1.6 to 4.5. Nonequivalence was also a problem, not just between the low-volume and standard tests, but also between the two clinical testing services – though here, again, the consumer test deviated most. Unexpectedly, inter-subject and inter-service variability had a significant impact on results, even within clinical testing services – a finding that may have implications for end-user interpretations of results, and ultimately for treatment decisions at the point of care.

What does this mean for DTC services? Should they be scrapped? Not necessarily; according to the report's authors, these kinds of tests cost substantially less than traditional services, and are often more accessible for patients with limited time or mobility. Rather, they suggest that it's important for test manufacturers and regulatory bodies to gain a greater understanding of the sources of variability, so that results can be interpreted in context and, eventually, testing technologies improved. As consumer testing doesn't appear to be a transient phenomenon, the goal should be to provide results as accurate and reliable as possible. *MS*

Reference

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Bacterial Biomarker Hope for Infants

Changes in the gut microbiome of infants who develop necrotizing enterocolitis may allow us to predict, and perhaps even treat, the disease

Necrotizing enterocolitis (NEC) – the name alone conjures up unpleasant images of intestinal disease and tissue death. It's worse still to realize that NEC affects approximately one in 10 premature infants, as well as many full-term babies with other health issues. "As a clinician, I am desperate to see new approaches toward prevention," says Barbara Warner, first author on a new study that reveals a potential microbial biomarker for the disease (1). "NEC is now the most common cause of mortality among preterm infants who survive the immediate perinatal period." Fortunately, Warner's new study may offer a means of tackling NEC – a disease where waiting until onset means it's already too late.

Warner and her colleagues at the National Institute of Allergy and Infectious Diseases examined stool samples from 46 premature infants who developed NEC and 120 age-, birthweight- and birthdate-matched infants who did not. Genetic sequencing and statistical analysis revealed that the NEC population had significantly higher proportions of Gammaproteobacteria in their gut microbiome, and lower proportions of Clostridia and Negativicutes. This discovery raises many more questions for the researchers. "We hypothesize that the potential driver(s) behind this microbial signature could be related to the microbial community itself, the host, or both," Warner explains. "Is it that specific pioneering microbes set the stage for subsequent

colonization patterns? What then would be the determinants of those pioneering microbes? Alternatively, could it be that the host response is a determinant of which microbial community evolves? Preterm infants have an immature immune system, and it is increasingly evident that the gut microbiome and host immune systems are in a close reciprocal relationship that may be impacted by that immaturity."

We can't be sure yet which came first – the chicken or the egg; the changes to gut microbiota or the disease. But as technology for microbial community profiling advances, we can certainly envision a use for these changes in the clinic. "A rapid turnaround test that screens this easily accessible analyte, stool, could be put into place. There are still challenges, however, including the longitudinal nature of the signature. Identification of infants at risk would require monitoring stool samples over time to follow the trajectory." The researchers are now investigating whether or not there are specific signatures related to disease severity and the need for surgical intervention – but while that work is ongoing, Warner says, "For the first time we can begin to examine microbially informed approaches for disease prevention. This bacterial signature offers, at the very least, a biomarker for soon-to-develop NEC. Moreover, we now have a set of plausible drivers, and little else in our toolbox to reduce incidence and improve the outcome (30–40 percent fatality rate) of NEC." The outlook for the disease has not changed meaningfully in four decades, but for pathologists who would like to see that change, Warner recommends, "Stay tuned to new methodology related to rapid microbial community profiles, rather than identification of any one specific organism as it relates to disease." *MS*

Reference

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Small Mutations, Big Impact

A new study reveals that screening for mutations in patients with advanced lung cancer can focus their treatment options and improve success rates

It's routine to recommend molecular profiling for patients with advanced non-small-cell lung cancer (NSCLC), with the goal of better understanding the origins and potential treatment susceptibilities of their tumors. Many such patients are screened for mutations in oncogenes, tumor suppressor genes and their regulators – but what does this screening actually accomplish? Is it feasible to screen every NSCLC patient who enters the healthcare system, and if it is, do the patients truly benefit from it?

The French Cooperative Thoracic Intergroup (IFCT) conducted a one-year study to answer these questions and optimize the standard of care for NSCLC patients (1). The study included 17,664 patients who were screened for mutations in genes including *EGFR*, *ALK*, *HER2*, *KRAS*, *BRAF* and *PIK3CA*, all known drivers of oncogenesis (2). Ultimately, about half of the patients assessed exhibited at least one mutation (see Figure 1) – a significant fraction worthy of further investigation. The study authors also established that the existence of a mutation affected both the choice of first-line treatment (in 51 percent of patients with alterations) and its likelihood of success (37 percent of patients with mutations achieved an overall response, compared with 33 percent of those without). And not only first-line treatment was affected; second-line treatment improved (17 percent of patients with mutations responded, vs. 9 percent of those without), first-line progression-free survival increased (10 months in those with mutations vs. 7.1 months in those without), and overall survival increased (16.5 months in those with mutations vs. 11.8 months in those without).

What can we take away from the French study? First, that routine molecular profiling of advanced NSCLC patients is feasible on a large scale. Second, and more importantly, that it's worthwhile to do this kind of screening. Because the detection of even a single genetic mutation can have such a significant effect on patients' treatment options, responses, and overall survival, examining the genetics of NSCLC patients may soon be a commonplace laboratory task. *MS*

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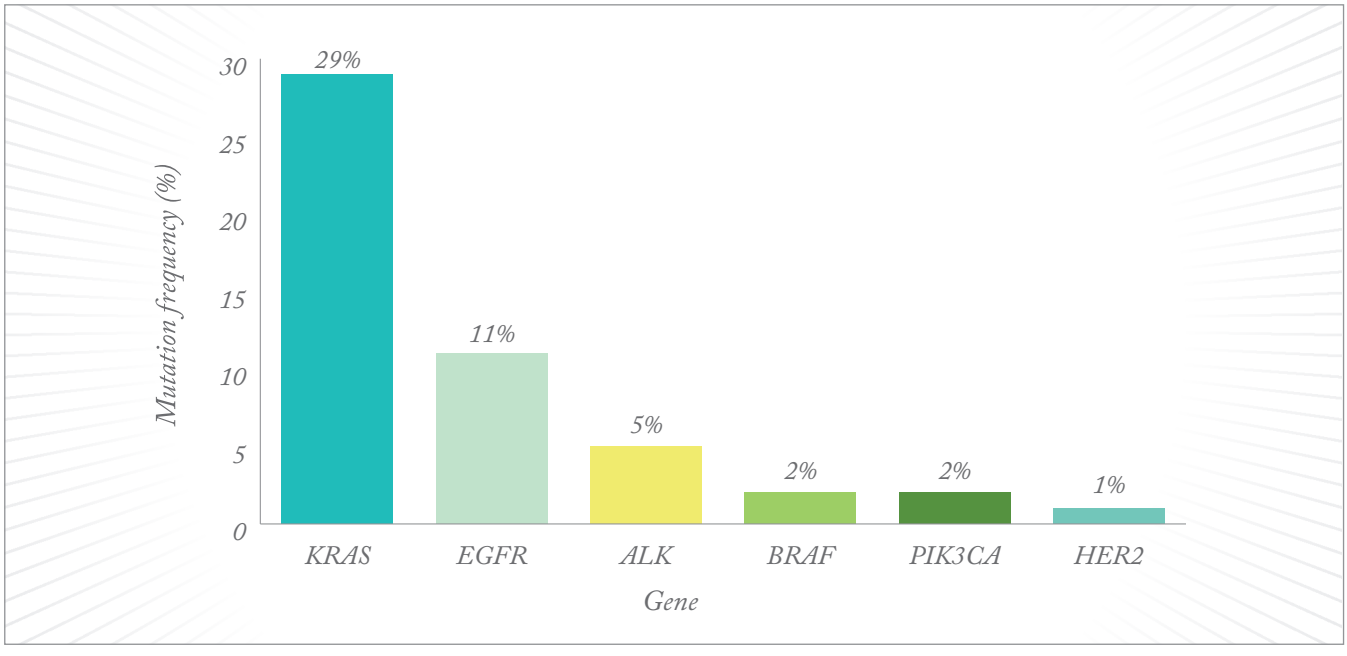

Figure 1. Frequency of genetic alterations in each screened oncogenic driver.

A PSA on PSA

The cost of prostate cancer screening can outweigh its benefits – but the right strategy can maximize the return on investment

The controversy over prostate cancer screening is a familiar one to pathologists. To screen, or not to screen? Many believe the costs and risks outweigh the benefits, and feel that more personalized – or more conservative – strategies to optimize screening are needed. But what are those strategies, and will they really help? Until now, experts have been uncertain. A new economic analysis from Seattle, which analyzes the cost-effectiveness of 18 different prostate-specific antigen (PSA) screening strategies, may hold answers (1).

The study involved creating a simulated cohort of 40-year-old men. The cohort was run through each of the 18 screening strategies, which varied by starting and stopping ages, screening intervals, biopsy referral criteria and choice of treatment practice (either contemporary, based on age and cancer stage and grade, or selective, wherein cases with Gleason score <7 or stage <T2a are treated only after clinical progression).

With contemporary treatment, the study found that only strategies with age-dependent thresholds or biopsy referrals for patients with PSA levels >10 ng/mL provided increased quality-adjusted life-years (QALYs). The only strategy that provided effective returns in terms of cost per QALY was screening patients aged 55 to 69 every four years. The more conservative selective treatment approach provided somewhat more benefit; all strategies were associated with increased QALYs and several (involving different age thresholds, screening intervals and referral criteria) were cost-effective.

What does this mean for the clinic? PSA screening can, according to this research, be cost-effective – but only if both screening and subsequent treatment are conservatively managed. It's important to ensure that such approaches don't overlook patients in need of more extensive care, though, and if the correct balance can be found, it's possible that the future will see fewer PSA tests ordered, fewer patients sent for biopsies, and an improvement in the cost-to-benefit ratio of prostate cancer screening. *MS*

Reference

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Respiration Inspiration

Can a new aerodynamics-inspired "sneezometer" that measures airflow with high speed and sensitivity offer a new tool for respiratory disease diagnostics?

David Birch, Senior Lecturer in Aerospace Engineering at the University of Surrey, UK and one of the Sneezometer's creators, seems to think so. He tells us more.

Who?

The University of Surrey Centre for Aerodynamics and Environmental Flow has a reputation for building new instruments to measure things nobody has measured before. This time, it's a special high-sensitivity spirometer known as a "sneezometer" (1). It was initially developed to address a tricky flow-measurement problem in aerodynamics, but a chance discussion with a health professional revealed the potential for the idea in medical care. At that point, Paul Nathan and I, along with our team, created an operational prototype in just three weeks! This project arose from Surrey's specialized expertise in wind tunnel measurement and is a great example of how fundamental research can sometimes result in incredibly beneficial technologies in an entirely unpredictable way. In this case, a simple tool developed for fundamental turbulence research has evolved into a medical instrument that could reduce costs for healthcare providers and affect the lives of millions of people suffering from chronic health conditions.

What?

The new spirometer measures the flow rate of air into and out of a patient's lungs with extremely high speed and sensitivity. In addition to its use as a conventional spirometer, the sneezometer is fast enough to accurately characterize coughing flow, something with which existing spirometers have difficulty. As a result, it could be used in the diagnosis of a variety of chronic and acute respiratory conditions including

David Birch demonstrates the Sneezometer.

asthma, obstructive sleep apnea (OSA) and hypopnea – all of which are highly prevalent and constitute a heavy burden to both healthcare systems and the lives of patients. Believed to be the most sensitive flow meter in existence, the sneezometer may have additional applications, for instance in the monitoring of neonatal infants or the training of elite athletes. It's currently being tested on healthy volunteers at King's College Hospital, London, but once approved for clinical use, we hope it will be used on patients of all ages for respiratory disease diagnosis (including remote diagnostics) and home monitoring.

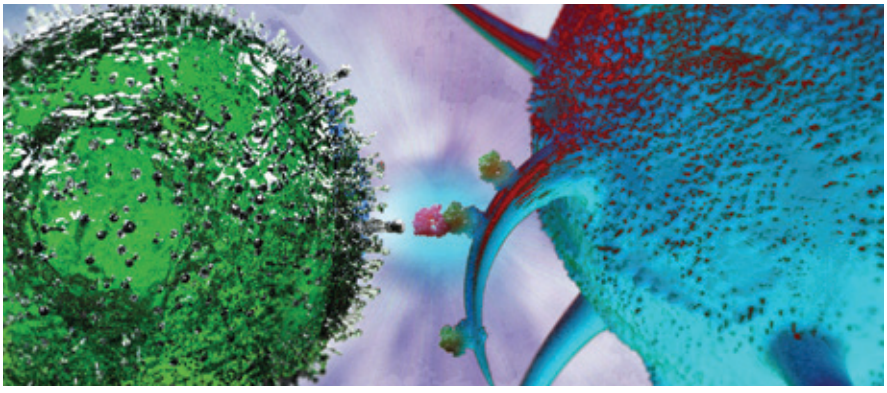
Why?

Our new instrument is much faster and more accurate than conventional spirometry systems, so we hope it will eventually replace those larger, more expensive devices. In addition, it's small enough to be portable (or even wearable), and its low cost would make it ideal for use in locations where conventional diagnostic tools are less available.

The sneezometer will make respiratory disease diagnosis easier, quicker and cheaper. It also provides much more detailed data than we've ever had before, so we don't know how far its potential could reach. Early (and exciting) indications from the testing at King's show that there may be a connection between the severity of an asthma attack and the airway turbulence. It may be that, as testing continues, our clinical colleagues find surprising new uses for our device!

Reference

1. *A Sutton, "World's first 3D-printed sneezometer' will help asthma patients breathe easy", (2016). Available at: http://bit.ly/1TXUg64. Accessed April 6, 2016.*



Tracing T Cells

New software can reconstruct T cell receptor sequences from RNA sequencing data, allowing us to track the origins and fates of immune cells

For all that we have learned about the immune system, much of it remains a mystery to us. What goes on behind the scenes in a T cell is a prime example of this – we don't know how these cells' fates are determined, nor do we understand the clonal relationship between different T cell types. But a new computational approach (1) from the Wellcome Genome Campus' Single-Cell Genomics Centre could shed new light on the immune response.

"TraCeR is a software tool that finds and reconstructs the recombined T cell receptor (TCR) sequences that are present within single-cell RNA sequencing data without needing any modifications to the RNA sequencing method," explains Mike Stubbington, who led the research. This tool allows researchers to do two things: first, they can find the sequence of both chains that comprise the TCR – essential to understanding what antigen that particular TCR can detect. Second, the huge possible diversity of TCR DNA sequences means that cells that share sequences are likely to be "siblings" that derive from the same original progenitor cell. This means that they can now track the family relationships between T cells within a sample. "Crucially, we get the information about TCR sequences in parallel to the RNA-seq gene expression profiles of each cell," says Stubbington. "This means that we can find out whether all the 'children' of a particular T cell do the same thing at the same time, and whether the antigen detected by a T cell can influence its fate choices."

The tool relies on the presence of recombined and expressed DNA sequences that occur naturally within cells of interest – which means that it can be used in B cells as well as T cells, a step the researchers plan to take soon. "This enables us to think about the relationship between T cell specificity and cellular choices. It also allows us to think about how sibling cells make different 'life choices' and perform different roles during an immune response." Unfortunately, the cost of TraCeR is currently prohibitive for routine clinical use, but if technological advances bring the price tag down, it might one day provide useful information about patient responses to vaccination or drug treatment in infectious disease, cancer or autoimmunity. *MS*

Reference

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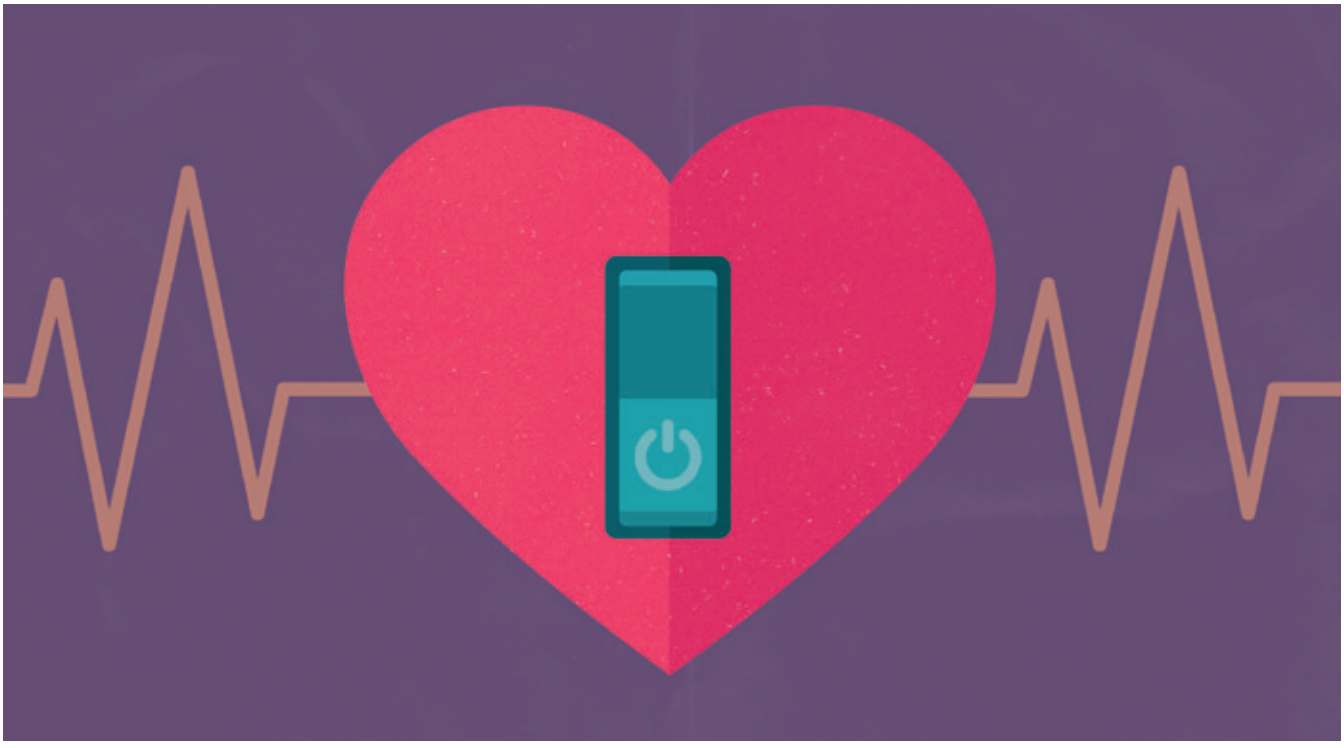

An On/Off Switch for Heart Attacks

ANGPTL4 mutations can reduce patients' risk of heart attack by up to 50 percent – so could neutralizing the gene offer a new route to prevention?

Picture "heart attack prevention" and no doubt what comes to mind is diet, exercise and healthy lifestyle choices. But what if we could simply flip a switch to lower the risk of coronary artery disease? A recent discovery by the German Heart Center at the Technical University of Munich brings us one step closer to that reality.

Medical director Heribert Schunkert and an international team of cardiologists examined nearly 200,000 people – both previous heart attack patients and healthy controls – and analyzed 13,715 genes for differences between the two groups (1). Of the four genes correlated with disease, *ANGPTL4*, which codes for the protein angiopoietin-like 4, was the best candidate for further investigation. Subjects with mutations in the gene exhibited significantly lower levels of blood triglycerides than those without. "Based on the genetic findings, *ANGPTL4* becomes a very strong candidate for therapeutic intervention, because loss of one allele of the gene caused no harm," explains Schunkert. "The same was true for genetic variants that came with lower activity of *ANGPTL4*. Indeed, aside from lower triglycerides and 50 percent lower risk of myocardial infarction, no 'side effect' was observed. Thus, neutralization of *ANGPTL4* might be a straightforward therapeutic approach."

It's already possible to measure patients' triglyceride levels in the clinic – and Schunkert emphasizes that the value of that measurement is underestimated, and that doctors and patients should focus just as much on it as on cholesterol. The normal function of *ANGPTL4* is to regulate lipoprotein lipase, an enzyme that breaks down triglycerides; neutralizing the gene decreases the levels of triglycerides in the blood and protects against coronary artery disease. Thus far, no negative effects have been observed in animal models treated with antibodies against *ANGPTL4*, and Schunkert hopes that a similar antibody will soon reduce the risk of heart disease in humans. *MS*

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Single-Gene Source for Schizophrenia?

Mutations in the *SETD1A* gene are rare, but significantly increase patients' risk of schizophrenia and neurodevelopmental disorders

Mental illnesses are tough to pin down – they're difficult to predict, tricky to diagnose, and challenging to treat. And yet they carry with them a huge burden, not only to those who suffer from them, but to society as a whole. The socioeconomic cost of mental illness in developed countries is estimated to be as much as 4 percent of GNP (1). That's a significant motivator to look at the nuts and bolts of neuropsychiatric disorders – and now, for the first time, researchers have conclusively identified a gene involved with one.

Mutations in the *SETD1A* gene almost never occur in the general population. They're not common in people with schizophrenia, either, arising in fewer than one in every 1,000 patients. But even though the gene is related to only a small percentage of patients, its mutations have been shown to increase the risk of schizophrenia 35-fold – and place patients at a higher risk of other disorders as well (2). "Our results already showed that damaging variants in *SETD1A* also cause early childhood developmental disorders, intellectual disability and epilepsy," explains Jeffrey Barrett, senior author on the paper. "This is a pattern that has been seen before in other genes, but never before including schizophrenia. So we see this link as an important piece of evidence about the interrelatedness of different neurodevelopmental disorders."

Unfortunately, the rarity of damaging *SETD1A* variants means that they probably won't lend themselves to screening or diagnostic use. That doesn't mean they won't be useful in the clinic, though. "Where we really hope to make a difference is by implicating new biology that might be useful for discovering new drugs for schizophrenia. *SETD1A* is part of pathway that regulates how DNA folds and unfolds during development to help switch on and off the right genes at the right times." Barrett points out that additional genes in the same pathway may also be involved in schizophrenia risk, so the pathway as a whole may offer an avenue for research into new therapeutics. *MS*

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In My View

In this opinion section, experts from across the world share a single strongly held view or key idea.

Submissions are welcome. Articles should be short, focused, personal and passionate, and may deal with any aspect of laboratory medicine. They can be up to 600 words in length and written in the first person.

Contact the editor at fedra.pavlou@texerepublishing.com

Empowering Pathologists

Digital pathology and tissue image analysis tools are essential to developing companion diagnostics and enabling pathologists to support clinical decisions, but there is still work to be done...

By Ralf Huss, Chief Medical Officer, Definiens

As personalized medicine in oncology continues to progress, companion diagnostics (CDx) are becoming ever more critical in the future of treatment and in its success. CDx help determine the patients most likely to respond to a therapy, but while detecting predictive biomarkers is a strong focus for the industry that develops these invaluable tools, identifying and developing robust CDx is still a very challenging process.

It starts with tissue analysis – and digital pathology, tissue image analysis and other data such as genomics – are essential. It is at this stage where the challenges begin. First, the biomarkers being identified and targeted for CDx can be inducible; thus, if a patient has already undergone treatment such as chemotherapy, the expression of those markers may be influenced and results will be impacted.

An additional primary obstacle currently is pathologists' inability to visualize the spatial distribution of immune cells and their correlation to the tumor. Understanding the spatial relationship between immune cells within the tumor setting is increasingly important in helping identify biomarkers in immuno-oncology, and it requires powerful tissue image analysis tools. As such, standardizing the use of digital pathology – which includes digitizing tissue slides and using automated tissue image analysis to extract relevant data about cancer cells, their behavior and their spatial relationships to other cells – is a key next step in advancing the diagnosis and treatment of cancers. While many are of the understanding that tissue image analysis simply automates routine tasks such as counting cells, there is so much more to these tools; they are able to dig deep into basic biology, providing the kinds of information traditionally found in quantitative liquid biopsies, but with the added morphological context that only tissue provides.

Today's digital pathology and image analysis tools enable pathologists to extract and quantify relevant information from digitized tissue images, helping them to not only identify markers, but also understand the correlation of different markers and their spatial relationships to one other. After gathering this tissue data, they can also view it in context with genomic and clinical data. This kind of "X-ray vision" assists pathologists in reading information beyond plain eyesight and coming to conclusions based on biologically relevant and clinically meaningful features in the tissue along with other important patient data. For pathologists in R&D, this means they have the ability to discover meaningful biomarkers; and for pathologists in the clinic, it means they can retrieve data that demonstrates how a patient will benefit from a particular treatment.

Tissue image analysis makes pathology an equally quantitative versus merely qualitative discipline. It's the only way to effectively develop tissue-based CDx, which will allow pathologists to successfully support clinical decision-making. And

significant technological advancements are being made.

The integration of multidimensional omics, or "multi-omics", which looks at a combination of genomic, proteomic, epigenomic, tissue phenomic and other relevant information as part of a big data approach to personalized medicine, is an important advancement. Multi-omics enables researchers and pathologists to rely on curated data correlations rather than isolated data sets to gain additional insights, answer research questions and make diagnostic and prognostic determinations.

Additionally, as tissue image analysis tools become more powerful and precise, they are also being evaluated for use in cancer immunoprofiling. Immunoprofiling helps provide a more personal picture of an individual patient's disease, how it is behaving and how the immune system is responding, and subsequently can support more effective clinical decision-making and patient treatment.

Ultimately, pathologists must be able to provide good guidance to oncologists on treatment options. In order to accomplish this, it is essential that industry formalizes the use of digital pathology, providing the tools for pathologists to make better decisions about disease diagnosis and prognosis than they are able to reviewing tissue samples by eye alone.

While in principle all intelligent components for the standardized use of digital pathology across the industry exist, in actuality there are still a number of challenges to overcome. Digital pathology by nature requires the scanning of available slides and the digitization of the tissue images, and while there are of course tools to do this, a lack of different resources across labs often creates limitations here. Additionally, since digital pathology platforms can digest millions of data points from tissue and other samples – which of course is where the opportunity lies for advanced CDx and clinical decision support – it can be a challenge to understand and analyze all of this data and then correlate it with patient outcomes. Finally, some skepticism across the industry – from companies to patients to payers – about this approach limits the growth of digital pathology.

There is still work to be done to convince all parties involved that digital pathology is crucial to the advancement of cancer drugs and diagnoses, but, as industry invests in more powerful tools and globally standardized lab and tissue testing procedures, pathologists will be in an even greater position to support oncologists and patients in making truly personalized treatment decisions.

On the Spot Diagnosis

Mobile devices promise a new future of point-of-care diagnostics for all

By Laura Lechuga, Professor, Nanobiosensors and Bioanalytical Applications Group, Catalan Institute of Nanoscience and Nanotechnology (ICN2), Barcelona, Spain.

Reality always surpasses fiction. Take, for example, the cult science fiction movie "Gattaca." In the film, the police identified citizens using instant genetic analysis on a mobile device. No one would have believed that it could actually happen. Years later, the fiction is turning into reality thanks to the latest advances in point-of-care (POC) devices, nanobiosensors, microfluidics, lab-on-chip and cellphone technologies. The idea of using your own smartphone as an instantaneous diagnostic device by just adding a few drops of your blood, saliva, urine or tears onto a biochip, is getting closer to reality every day – and it is a concept I find fascinating.

Such is their utility that POC technologies are applicable across a broad range of healthcare contexts – from preventive medicine to advanced personalized and precision medicine. Importantly, they open a window of hope for economically disadvantaged countries and low-resource environments, where most of the population does not have access to hospitals or clinical labs (but they have a cell phone).

POC devices can enable quick, simple and cost-effective identification of many diseases at a very early stage. They can identify conditions such as cancer, diabetes, stroke, pneumonia, hepatitis A, HIV, malaria or tuberculosis, including drug-resistant strains, among many other pathologies. They can provide sensitive detection of diseases and screen metabolic disorders and infectious diseases, or support adherence to treatments.

A typical POC device will identify and quantify disease biomarkers in bodily fluids due to a nanoscale biomolecular interaction between the target biomarker and its specific bioreceptor (on a biochip). How does it work? The procedure is generally very simple: the patient extracts the biochip

(specific for one disease or for a panel of them) from a sealed package, aggregates the sample (a few drops of a bodily fluid), and inserts the biochip in the mobile POC device. A specific biomolecular interaction will occur, resulting in a physical or chemical change whose detection enables identification of the disease. Measurements typically take a few seconds or minutes. The data can be read and processed using a dedicated app, which will diagnose the medical condition, suggest the right treatment or connect the patient with their doctor or directly with emergency services, if required.

In my view, the ideal POC device should be disposable, require no external power source, be able to deliver the result in less than five minutes, allow for the analysis of several biomarkers in the same fluid sample, and should cost less than US$1. Academic research groups, industry, governments and policymakers are aware of these major and rapid technological developments. Although the enabling technologies exist, the main challenge is the integration and connection of all of these in compelling, portable POC platforms.

Notwithstanding the technological barriers and challenges, global market estimates have grown from US$1.6 billion in 2013 to $5.6 billion in 2019 (according to Transparency Market Research), which is driving significant commercial interest and major competition. Also, several important prizes, such as the Qualcomm Tricorder Xprize (US$10 million) (1), the UK Longitude Prize (£10 million) or the EU "Horizon Prize for better use of antibiotics" (€1 million), the latter two aimed at solving the problem of global antibiotic resistance, indicate why we need revolutionary diagnostic tools.

I anticipate a frantic struggle among the major players to develop the first POC smartphone for routine use in our daily lives. Such mobile health monitoring tools will open the door to a new world where preventive healthcare and truly personalized medicine are routine. The technology is already here. But are we ready for the next diagnostic revolution that places healthcare into the palm of your hand?

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Undervalued and Underused

Ultrasound guided fine needle aspiration biopsy cytology streamlines and improves cancer diagnosis

By Susan Rollins, Associate Professor of Pathology and Medical Director, Outpatient Cytopathology Center, East Tennessee State University, Johnson City, USA

Fine needle aspiration (FNA) biopsy should be used to greater advantage for diagnosis and management of patients. Currently, many pathologists use it to document recurrent disease in a patient with known cancer, and others use it to drain cystic lesions. But due to a lack of training for pathologists in FNA cytology, many clinicians are actually unaware of the diagnostic power of FNA biopsy; many see excisional biopsy or core needle biopsy as the biopsy procedure of choice, because, historically, tissue is king. Consequently, surgeons will receive requests to obtain large tissue biopsies at the expense of the patient, both financially and in terms of morbidity.

Medicine has entered a new era, though, because of the revolutionary progress in human genomics. And as a result, molecular techniques are being used to diagnose, treat, and monitor treatment response and disease progression of an ever-increasing number of diseases. In my opinion, FNA biopsy is extremely well suited to procuring samples for molecular studies and deserves consideration as a first-line technique whenever possible. My reasons? Cells obtained by FNA are ideal for a multitude of ancillary studies and they may be superior to tissue biopsy specimens for such diagnostic tests. Indeed, more use of FNA biopsy during initial workup of mass lesions could reduce patient time and medical costs, and improve post-biopsy recuperation. And this would be accomplished without jeopardizing care!

Cytology specimens can be used for immunohistochemical (IHC) stains, flow cytometry, fluorescence in situ hybridization (FISH) and molecular tests for biochemical and genetic defects. For example, let's say a patient has an enlarged supraclavicular lymph node. In such a case, FNA yields cells compatible with adenocarcinoma, and IHC stains are most consistent with a lung primary. Molecular studies are performed on the sample and, based on the molecular results, the patient proceeds directly to oncology for treatment. Another scenario

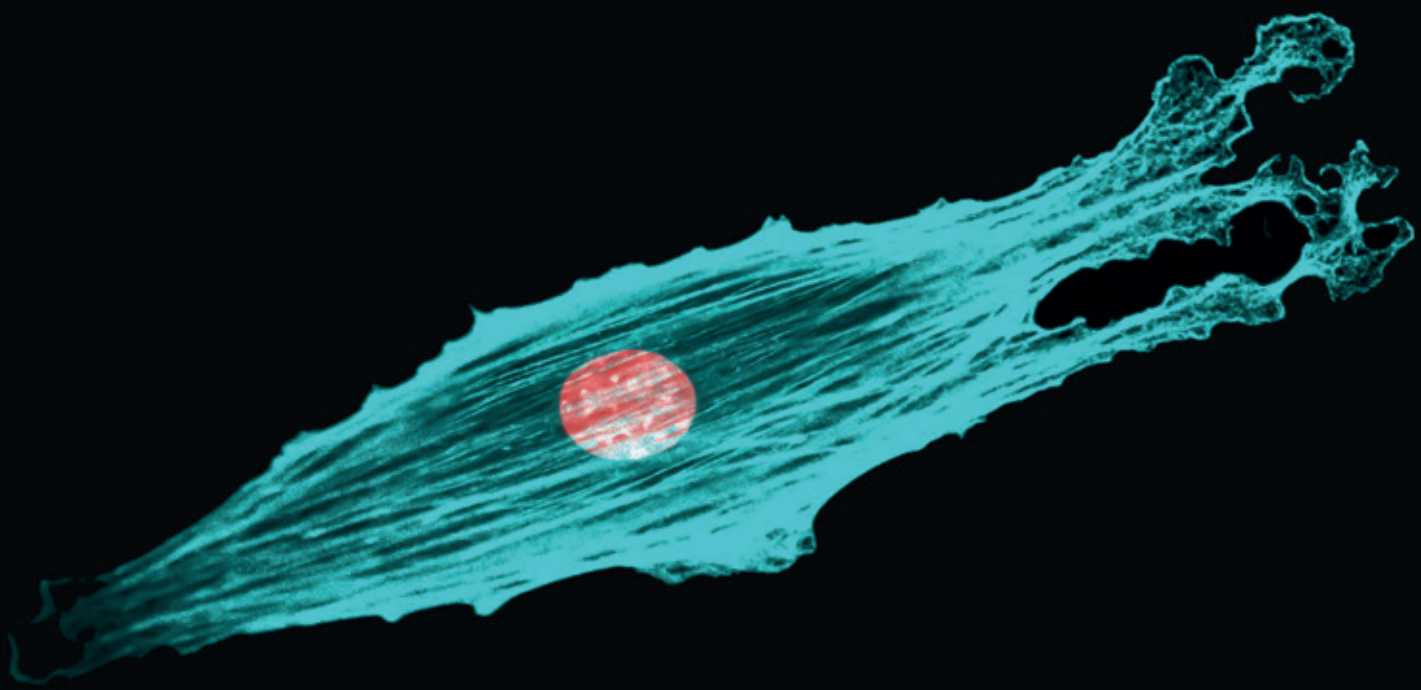
could be a patient with an enlarged cervical lymph node demonstrating squamous cell carcinoma on aspiration biopsy. A needle rinse specimen can be tested for high-risk human papilloma virus (HPV) subtypes and therapy instituted on the cytology results. All of this information is obtainable using a narrow gauge needle with a simple, non-invasive, economical, quick outpatient clinic procedure.

Current practice for FNA biopsy in many centers is to obtain the specimen in the radiology department and then send it to pathology. Under this paradigm, though, there is no indication if a specimen contains the requisite number of cells to qualify as an adequate specimen until after the patient has left the clinic. If the biopsy cellularity is insufficient for diagnostic evaluation, or if the pathologist needs additional specimen for ancillary testing, the patient must return for a second biopsy. This is not an effective use of medical resources or the patient's time. Additionally, some practices require the availability of a pathologist or cytotechnologist when the biopsy is done for "rapid on site evaluation" (ROSE) for specimen adequacy. ROSE is effective for obtaining a satisfactory sample but has limitations; it is time-intensive for pathology and reimbursement is low. Also, unless a pathologist is available, immediate decisions about triaging the specimen for studies such as flow cytometry, cultures, or molecular studies, are hindered.

A partial solution to the above problem is training pathologists in ultrasound guided FNA (USFNA) cytology. Pathologists with good hand-eye coordination can learn the USFNA technique reasonably quickly and this skill is immensely useful for any pathologist performing FNA biopsies. Ultrasound guidance allows for selective sampling of masses, which can sometimes make the difference between an adequate and inadequate sample. Ultrasound also gives the pathologist useful information about the lesion sampled such as shape, size, echogenic pattern and blood flow; this information can be integrated with the cytologic findings to render a more precise diagnosis or give guidance for follow-up care. USFNA by cytopathologists of superficial body sites including thyroid, superficial lymph nodes, salivary glands, breast and soft tissue, is within our scope of practice.

"Pathologists with good hand-eye coordination can learn the USFNA technique reasonably quickly and this skill is immensely useful for any pathologist performing FNA biopsies."

For those cytopathologists interested in optimizing FNA biopsy, particularly in this era of personalized medicine, I encourage them to explore the realm of USFNA. Not only will there be fewer specimens of insufficient cellularity for diagnosis with the technique, but patients will also benefit from the most appropriate testing on their FNA specimen.



Cells on the (Invasion) Front Lines

Long thought to be a key component of the metastatic process, researchers are now questioning long-held beliefs of the role of epithelial-to-mesenchymal transition

By Michael Schubert

The diagnosis and treatment of cancer has moved ahead by leaps and bounds – except when it comes to metastatic disease. Cancer that has spread beyond its origins remains the leading cause of death from the disease, according to the World Health Organization,, and although it's a major focus of research, we know little more about its mechanisms today than we did a decade or more ago. This is especially true when it comes to the epithelial-to-mesenchymal transition (EMT), a key component of the metastatic process… or is it?

The debate over EMT's significance in cancer spreading is a fierce and ongoing one, largely due to the difficulties inherent in observing and understanding it. Why are researchers so convinced of EMT's role in metastasis? It's well known that mesenchymal cells are more capable of escaping the primary tumor, and of taking up residence in distant sites. But the evidence against EMT-driven metastasis is mounting, too – most cells in metastatic lesions exhibit epithelial, not mesenchymal, characteristics. Some scientists refer to the reverse process, mesenchymal-to-epithelial transition (MET) to explain this behavior, but others aren't so sure. At the root of the confusion is a lack of evidence. Until the entire metastatic process – local invasion, intravasation, circulation, arrest and extravasation, proliferation, and angiogenesis – is observed in mesenchymal cells, the role of EMT in metastasis remains an open question.

Despite the debate, many researchers simply take EMT's role in metastasis as read. Searching the PubMed database for "EMT and metastasis" brings up 3,675 publications, and even Wikipedia – the first port of call for most non-experts without access to peer-reviewed articles – boldly states, "EMT and MET form the initiation and completion of the invasion-metastasis cascade." There's little indication of doubt, and yet, recent studies are threatening to completely overhaul the research community's view of EMT and metastasis. Exactly how do these two aspects of cancer biology interact? The quest to understand is more intense than ever, thanks to groundbreaking new data from research groups whose conclusions go against the grain.

PROCEED WITH CAUTION

Two recent studies on EMT may revise the field's understanding of the process – but it's important to keep in mind both the breakthroughs and the limitations of the work

By Shyamala Maheswaran

For many years, cancer researchers have believed that metastasis relies on the transition of tumor cells from an epithelial to a mesenchymal phenotype. Even after tumor analysis revealed that the cells of secondary cancers exhibit epithelial characteristics, this was ascribed to a reversal of the transition – from mesenchymal back to epithelial phenotype. Why has this belief persisted so strongly despite uncertainty and debate – and why have the recent papers by Fischer et al. (see page 26, "Tracking the Transition") and Zheng et al. (see page 27, "The PDAC Key") had such an impact on the research landscape?

EMT is an embryonic process required for proper development. It has been observed in tissue culture upon expression of various transcription factors, and following treatment with different cytokines. In vitro, EMT is associated with increased cell migration and invasion. In many cases, the increased invasion observed in vitro translates into increased metastasis in mouse tumor models. But clinical evidence supporting EMT in human tumors has been somewhat limited, due to the difficulty in distinguishing mesenchymally transformed cancer cells from reactive fibroblasts within a tumor. This has led to some debate regarding the importance of EMT in tumor dissemination in the clinical setting. That's where the two new studies may shed light.

Pros and cons

EMT is reversible; it's currently believed that epithelial cells transition into a mesenchymal state, then revert to the epithelial state upon reaching the distal site. The plasticity and transient nature of EMT has made it difficult to follow these cells from the time they transition to a mesenchymal state, through invasion into the blood, and to the point of colonization at distal sites. The two studies reported in Nature are particularly interesting because they both addressed this problem, albeit using very different approaches. Fischer et al. used green fluorescent protein (GFP) expression as a proxy for mesenchymal transition and traced lineage-switched epithelial tumor cells from inception to metastatic colonization in two different mouse mammary tumor models. Zheng et al. knocked down the EMT-inducing transcription factors Snail and Twist in the pancreatic epithelium of mice so that they could monitor the consequences of EMT in the metastatic dissemination of pancreatic tumor cells. These approaches allowed definitive, real-time monitoring of the tumor cells and concluded that EMT is dispensable for metastatic colonization, but plays a role in drug resistance.

> "These approaches allowed definitive, real-time monitoring of the tumor cells and concluded that EMT is dispensable for metastatic colonization, but plays a role in drug resistance."

That doesn't mean that these studies are without limitations (1). First, EMT relies on a complex signaling network that involves multiple transcription factors and signaling proteins, in some instances with redundant functions. Whether lineage-tracing studies with single genes can accurately mimic this complex process is unclear. Second, cancer progression involves a continually evolving genomic and epigenetic landscape, so it's unlikely that mouse tumor models driven by only a few oncogenic events fully recapitulate this process. The studies certainly show that EMT is dispensable for metastasis, but readers must recognize the limitations of the mouse models.

Interestingly, in the mouse model generated by Fischer et al., epithelial tumor cells that switch to a mesenchymal state are permanently marked with GFP expression, and illustrate that a small subset of such cells do indeed spontaneously transition into a mesenchymal state (although it isn't required to drive overt metastasis). The prevalence of green cells following drug treatment suggests that cells with a history of EMT, regardless of their current state, are more resistant to drugs. The mechanism by which EMT increases cell survival under adverse conditions is not yet known – but perhaps our new understanding of EMT will provide a springboard for further research.

Old theories have been challenged – now what?

The new studies tell a very different story compared to what has been shown before. Why? No one can say for certain, but the EMT models used for in vitro research represent powerful induction of the transition by EMT-inducing transcription factors and cytokines. Spontaneous EMT in clinical specimens might be much more subtle, and could account for – or at least contribute to – the discrepancy between these two studies and those that have previously been reported. Another consideration is that EMT relies on the activation of complex and sometimes redundant signaling modules, an aspect not reflected by the mouse models used in the Nature studies. Although those models do show that EMT is dispensable for metastasis, the findings need to be evaluated within the context of the complexity of tumor progression, which involves an ever-evolving genomic and epigenetic landscape.

EMT is an attractive concept to define the process of metastasis: it involves loss of cell-cell interaction and gain of cell motility. But there are other cellular mechanisms of tumor dissemination, like collective epithelial cell migration or tumor microemboli, that may drive the spread of cancer. And metastasis isn't the end of the story – EMT is also emerging as an important contributor to drug resistance, a phenomenon supported by the findings from both Nature papers. In my own recent work, my colleagues and I demonstrated drug-induced shifts in the epithelial and mesenchymal tumor populations of breast cancer patients. So although the new findings raise questions about EMT's role in metastasis, they also show that the transition does occur in tumors – and not without a purpose, as cells that switch lineage are more resistant to drugs. It's now critical to gain further insight into the molecular nature of this process, so that we can use that information to research better treatments and more accurate prognoses.

As the field moves toward a more complete understanding of the tropism exhibited by tumor cells shed into blood and the role of EMT in drug resistance, I have one word of caution for researchers and clinicians alike. It's important to carefully evaluate what we learn about metastasis from cell culture and mouse models against both human clinical samples derived from repeat biopsies or tumor cells circulating in the blood and freshly established tumor cell cultures. By keeping an open mind to both new information and the limitations of pioneering studies, we can ensure that we're able to focus on the "big picture" of how cancer metastasis happens and what we can do to combat it.

Shyamala Maheswaran is Associate Professor of Surgery at Harvard Medical School and Assistant Molecular Biologist at the Center for Cancer Research, Massachusetts General Hospital, Boston, USA.

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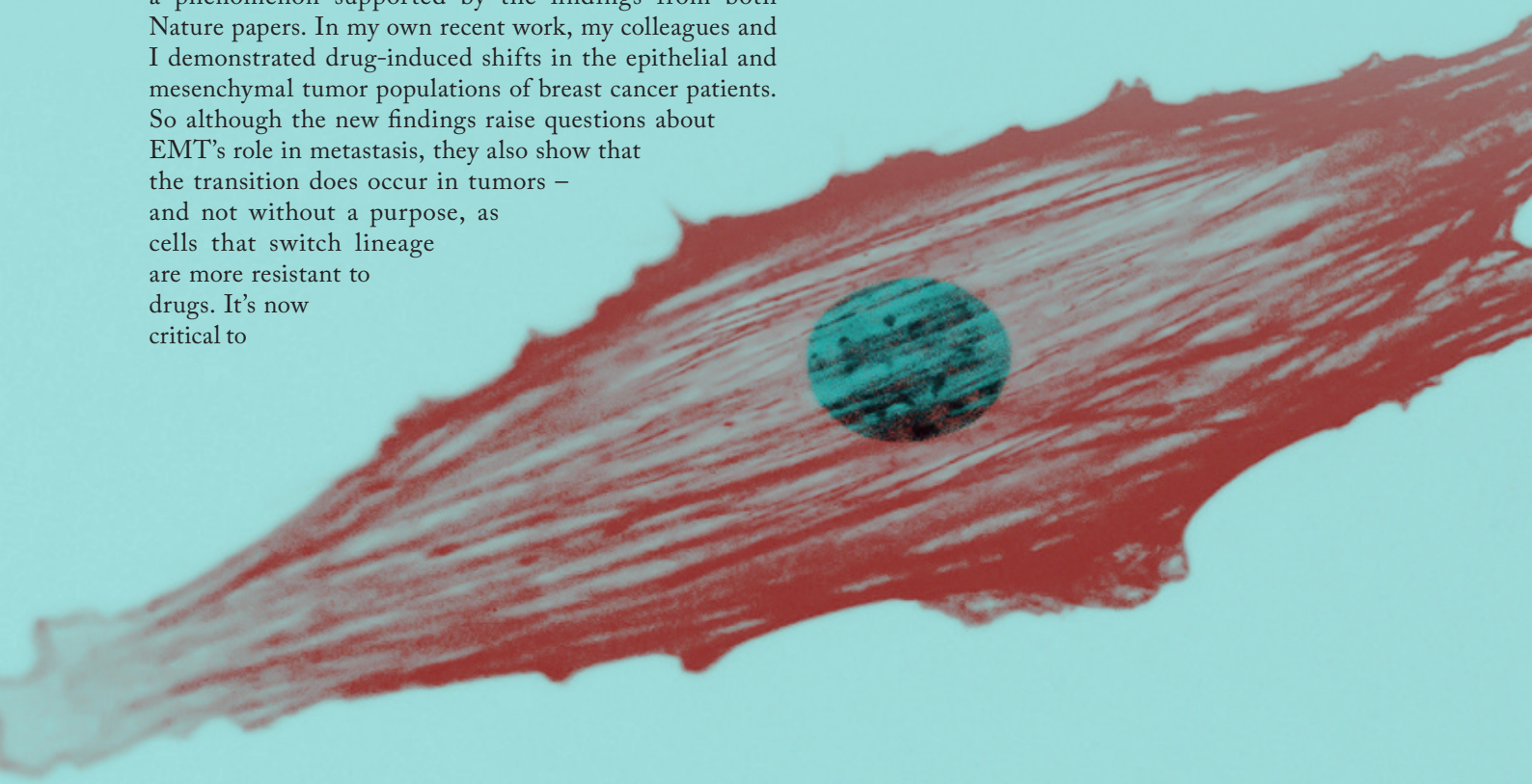

TRACKING THE TRANSITION

A triple-transgenic mouse model allows researchers to trace the lineage of EMT tumor cells, and reveals the transition's surprising lack of significance in metastasis

At Weill Cornell Medical College in New York City, Dingcheng Gao's research group studies cell and developmental biology. Recently, he and his colleagues published a paper outlining their research into EMT (1). They identified a key difficulty with our understanding to date: namely, that there's no way to track transient and reversible EMT phenotypes in living organisms. Without that ability, we can't find out whether or not cells are indeed undergoing EMT to initiate metastasis, then undergoing MET to return to an epithelial phenotype.

So Gao and his colleagues generated a triple-transgenic mouse model known as *MMTV-PyMT/Rosa26-RFP-GFP/Fsp1-cre*, or tri-PyMT. The mouse has three special attributes: an oncogene (PyMT or, in some cases, Neu) driven by the *MMTV* promoter; a recombinase (Cre) driven by the mesenchymal-specific *Fsp1* promoter; and two fluorescent proteins, red and green, each under separate control. The fluorescent proteins combine to form an irreversible color switch system – so once a cell has undergone EMT (and acquired green fluorescence), it's incapable of reverting to red fluorescence. This means that it's easy to see which cells have made the transition from epithelial to mesenchymal, even after they have transitioned back to epithelial characteristics.

"We wanted to find direct evidence in vivo to prove the EMT/MET hypothesis in metastasis formation," explains Gao. "Therefore, we established the EMT lineage tracing model using a permanent fluorescent marker switch to trace the reversible EMT process." But the team were in for a surprise. The cancer cells of the mice, which developed primary breast tumors followed by spontaneous lung metastases, didn't show the expected results. In fact, they showed exactly the opposite: none of the secondary lesions changed color following the natural progression of lung metastasis. The lack of color switching indicates that *Fsp1*, the mesenchymal promoter designed to permit green fluorescence, was never activated – and thus, that the metastatic cells may never have undergone EMT. Furthermore, inhibiting EMT with the use of the microRNA miR-200 prevented red-to-green color switching – but had no effect on the ability of tumor cells to metastasize.

"Cancer cells are capable of metastasizing through other mechanisms, such as collective invasion and random dissemination," says Gao. He cites a recent report by Cheung et al. in which the authors traced the lineage of metastatic tumors and showed that seeding by cell clusters, rather than by single cells, can result in polyclonal metastases (2). Collective invasion is typical of carcinomas like those often found in the breast or lung, and challenges the belief that metastases arise from single "escaped" tumor cells that undergo EMT. But if EMT isn't the key player in cancer dissemination, then what is?

"We've observed that EMT is a relatively rare event in primary tumors," says Gao. "Even though EMT tumor cells gain some anti-apoptosis properties that may help them survive in circulation, these advantages are accompanied by a downside – a decreased ability to proliferate. In general, metastasis is a very inefficient process for tumor cells. In our experiments, the rare cells that had undergone EMT were easily outnumbered by the epithelial cells, not just in the primary tumor, but also in the circulation and metastatic lesions." So if EMT is costly for tumor cells and most metastatic cells show no evidence of needing to undergo it despite its potential survival advantage, what is its purpose in the tumor?

> "Our results suggest that tumor cells that undergo EMT are more resistant to chemotherapy than non-EMT cells."

The second part of the Cornell paper offers an answer. Evidence from previous studies has suggested a link between EMT and chemoresistance – most notably in residual breast cancer, where the remaining tumor cells display mesenchymal characteristics (2). Gao and his team decided to investigate this link by treating their tri-PyMT mouse models with cyclophosphamide. Even during the initial treatment phase, green fluorescent (mesenchymal) cells were less proliferative – but also less apoptotic – than epithelial cells, indicating lower susceptibility to chemotherapy. But in metastatic lung tumors, the effect stood out even more. The mesenchymal cells outnumbered the epithelial population by almost three to one, and made notable contributions to five of the 17 total metastatic lesions (in contrast to untreated mice, where no lesions contained a significant mesenchymal cell population). "Post-EMT tumor cells showed a greater ability to survive chemo treatment," Gao summarizes. "This won them a better chance to develop into metastatic lesions."

"Our results suggest that tumor cells that undergo EMT are more resistant to chemotherapy than non-EMT cells. More importantly, we have observed a significant contribution of these EMT tumor cells to metastasis formation under chemotherapy conditions. Therefore, targeting EMT tumor cells may provide novel therapeutic approaches to overcome

chemoresistant metastasis." Gao thinks this is a vital piece of knowledge in the clinic. "Given that most patients with advanced-stage tumors are treated with chemotherapy, it's important to evaluate the EMT status of their tumors. Patients whose cells have undergone the transition would benefit from EMT-targeting therapy approaches." Not every cancer recurrence is based on EMT, though, and we now know that most metastases may not be rooted in that transition, either. As a result, pathologists need to closely examine tumor characteristics on a case-by-case basis to determine how best to treat and monitor each patient.

Of course, there's much still to be learned about the nature of metastasis. "One immediately attractive question," says Gao, "is whether the metastatic epithelial tumor cells differ in other characteristics from the majority of cells in the primary tumor. Characteristics like CK14 expression, multiple clonality, and other potential mechanisms in metastasis need to be further investigated." For his part, he and his laboratory are currently focused on developing novel strategies for targeting EMT tumor cells, with the hope of one day finding a way to overcome cancer chemoresistance.

Dingcheng Gao is Assistant Professor of Cell and Developmental Biology in Cardiothoracic Surgery at Weill Cornell Medical College, New York, USA.

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THE PDAC KEY

Pancreatic cancer cells don't seem to rely on EMT for metastasis – but it plays a key role in their ability to resist our best chemotherapy options

At the same time, hundreds of miles away in Houston, a group of researchers from the MD Anderson Cancer Center, Baylor College of Medicine and Rice University were collaborating on a closely related piece of work. Using specialized mouse models of pancreatic cancer with impaired EMT, Raghu Kalluri and his colleagues were investigating the transition's role in mediating metastasis and chemoresistance. The unexpected conclusion they reached mirrored the one from Dingcheng Gao's group – namely, that pancreatic cancer, like breast tumors, can metastasize without undergoing EMT (1).

The group began by creating transgenic mouse models of pancreatic ductal adenocarcinoma (PDAC) in which either Snail or Twist, two of the transcription factors responsible for inducing EMT, were knocked out. Deleting the *Snai1* and *Twist1* genes had no effect on the development or appearance of pancreatic tumors, but the researchers noted a significant decrease in cells undergoing EMT. Immunolabeling of the primary tumor showed far fewer epithelial cells that expressed either αSMA (a mesenchymal marker indicating EMT-positive status) or Zeb1 (another EMT-inducing transcription factor similar to Snail), and global gene expression profiling revealed a decrease in the expression of EMT-associated genes. What was increased, on the other hand, was the degree to which cancer cells proliferated when the transition was suppressed. With no change in the timing of tumorigenesis and local invasion, it's clear from these experiments that PDAC doesn't rely on EMT to initiate and progress.

But metastasis is at the heart of the question. Do these cancers rely on EMT in order to spread to distant areas of a mouse's – or a patient's – body? The researchers compared circulating tumor cells in control and EMT-suppressed mice and found that the numbers were unchanged. Histopathology and immunostaining in livers, lungs and spleens (the major target organs of metastasis) revealed approximately the same frequency of cancer spreading in both groups – and, when examined more closely, the metastases all proliferated at about the same rate and were largely negative for EMT-inducing factors Twist, Snail, Zeb1 and αSMA. The take-home message? Removing EMT from the equation doesn't affect the cells' ability or inclination to metastasize.

So it appears that the Texas group's pancreatic tumors behave much like the Cornell group's breast cancers. Is the same true of the cells' ability to survive chemotherapy? Previous studies have established a link between EMT and gemcitabine resistance in PDAC (2–4). "Gemcitabine works primarily on cancer cells that are dividing or proliferating. When cancer cells suspend their proliferation – such as when they launch an EMT program – then anti-proliferation drugs like gemcitabine do not target them well," says Kalluri (5). The next step, then, was to test sensitivity to the drug in cells with suppressed EMT. The researchers administered gemcitabine to control, Snail- and Twist-knockout

mice and discovered that, with the EMT-inducing factors removed, the chemotherapy-treated animals showed improved histopathology and survival. This held true across different mouse models of pancreatic cancer, all of which showed better responses to gemcitabine after EMT suppression – decreased tumor burden and proliferation, increased cancer cell death, and extended survival times.

"We found that EMT program suppressed drug transporter and concentrative proteins, which inadvertently protected these cancwer cells from anti-proliferative drugs such as gemcitabine," says Kalluri. "The correlation of decreased survival of pancreatic cancer patients with an increased EMT program is likely due to their impaired capacity to respond to chemotherapy, leading to overall poor prognosis and higher incidence of metastasis." (5)

Are there other possible explanations? The research still has gaps; it's possible that other EMT-inducing transcription factors are replacing Snail and Twist in knockout mice, or that EMT suppression from birth (as in the mouse models) has a different effect to EMT suppression only at or after the onset of disease. It doesn't look like the transition plays a significant role in PDAC metastasis – but in order to make that statement conclusively, more research, and probably more fierce debate amongst researchers, is needed.

But at the moment, the findings are fairly clear with respect to chemoresistance, and it seems clear that – by reducing proliferation and decreasing the expression of genes involved in transporting and concentrating drugs – the transition confers resistance to treatment and thus compromises patient survival. What does that mean for the clinic? Ultimately, that establishing a patient's EMT status may provide insight into the potential for treatment – and that although treatments targeting the transition may not prevent metastasis, they offer a potential way of enhancing the effectiveness of existing therapies.

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3. *T Arumugam et al., "Epithelial to mesenchymal transition contributes to drug resistance in pancreatic cancer", Cancer Res, 69, 5820–5828 (2009). PMID: 19584296.*
4. *K Zhang et al., "Knockdown of snail sensitizes pancreatic cancer cells to chemotherapeutic agents and irradiation", Int J Mol Sci, 11, 4891–4892 (2010). PMID: 21614180.*
5. *MD Anderson, "Study reveals why chemotherapy may be compromised in patients with pancreatic cancer", (2015). Available at: http://bit.ly/1VMjFkh. Accessed April 11, 2016.*

RESEARCH TIMELINE

1995

An overview of epithelio-mesenchymal transition

ED Hay

The epithelial-to-mesenchymal transition produces a mesenchymal tissue type in higher chordates. It's a central process for embrogenesis. But mesenchymal cells, unlike epithelial ones, can invade and migrate through the extracellular matrix – meaning that EMT has the potential to create invasive metastatic carcinoma cells. E-cadherin gene transfection can convert mesenchymal cells back to epithelial phenotype.

Acta Anat (Basel), 154, 8–20.

2007

Snail, Zeb and bHLH factors in tumour progression: an alliance against the epithelial phenotype?

H Peinado et al.

Snail, Zeb and some basic helix-loop-helix (bHLH) factors induce EMT and repress E-cadherin expression. These changes are associated with tumor progression. As a result, further research into these EMT-inducing factors may ultimately have clinical implications, with the potential for targeted treatments that prevent EMT and restore E-cadherin expression.

Nat Rev Cancer, 7, 415–428.

2008

The epithelial-mesenchymal transition generates cells with properties of stem cells

SA Mani et al.

The induction of EMT in human mammary epithelial cells results in the acquisition of not only mesenchymal traits, but also properties associated with stem cells (like increased expression of stem-cell markers or the ability to form mammospheres). Stem-like cells and post-EMT cells exhibit similar behaviors and express similar markers, and post-EMT cells are more efficient at forming mammospheres, colonies and tumors.

Cell, 133, 704–715.

2010

EMT, cancer stem cells and drug resistance: an emerging axis of evil in the war on cancer

A Singh, J Settleman

"EMT induction in cancer cells results in the acquisition of invasive and metastatic properties." The transition can also contribute to the emergence of cancer stem cells and drug resistance. It's possible that reversible epigenetic changes associated with chemoresistance may depend on the differentiation state of the tumor – and thus on cancer cells' stem cell-like characteristics or EMT status.

Oncogene, 29, 4741–4751.

2011

Cancer stem cells and epithelial-to-mesenchymal transition (EMT)-phenotypic cells: are they cousins or twins?

D Kong et al.

Cells that have undergone EMT share molecular characteristics with cancer stem cells and are associated with tumor aggressiveness and metastasis. "The acquisition of an EMT phenotype is a critical process for switching early stage carcinomas into invasive malignancies, which is often associated with the loss of epithelial differentiation and gain of mesenchymal phenotype."

Cancers (Basel), 3, 716–729.

2014

Twist1-induced dissemination preserves epithelial identity and requires E-cadherin

ER Shamir et al.

What are the minimum molecular events necessary to induce the dissemination of epithelial cells? Expression of EMT induction factor *Twist1* resulted in rapid dissemination, along with changes to extracellular compartment and cell–matrix (but not cell–cell) adhesion genes. The cells were unexpectedly able to disseminate with membrane-localized β-catenin and E-cadherin (whose knockdown strongly inhibited the process). Therefore, dissemination can occur without loss of the epithelial phenotype – indicating that cancer metastasis might also occur without EMT.

J Cell Biol, 204, 839–856.

Now

Epithelial-to-mesenchymal transition is not required for lung metastasis but contributes to chemoresistance

KR Fischer et al.

Nature, 527, 472–476.

Epithelial-to-mesenchymal transition is dispensable for metastasis but induces chemoresistance in pancreatic cancer

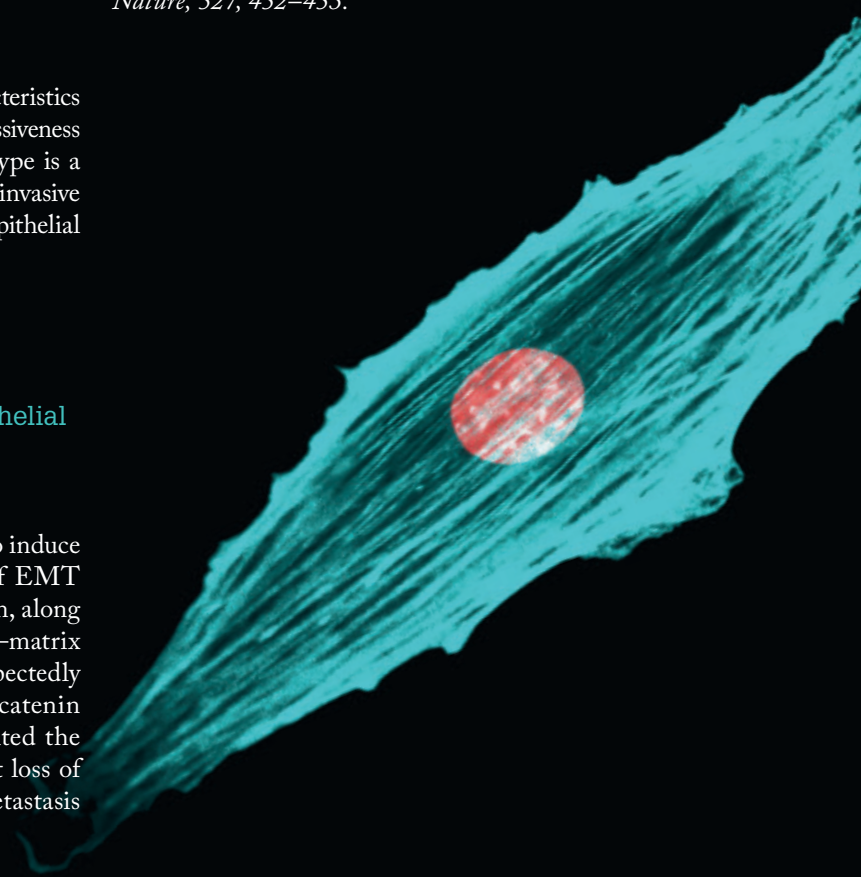
X Zheng et al.

Nature, 527, 525–530.

Cell fate: Transition loses its invasive edge

S Maheswaran, DA Haber

Nature, 527, 452–453.



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The Core of the Problem

Next generation tissue microarrays overcome punching inaccuracies and bring the technique into the era of precision medicine

By Inti Zlobec

As the era of precision medicine dawns, the role of the pathologist is extending beyond diagnosis to include the interpretation of molecular tissue biomarkers. The ability to detect protein, RNA and DNA alterations in cancer now helps us to tailor prognoses and predict therapy responses. As a consequence, biomarker research has exploded over recent years – and with it, the number of studies using tissue microarrays (TMAs) as a tool for biomarker evaluation and validation. Essentially, the TMA is a tissue archive constructed by transferring small tissue cores, typically 0.6 or 1.0 mm in diameter, from a "donor" tissue block into a "recipient" block. Repeated multiple times, a recipient TMA block can carry up to 500 different tissue cores from multiple tumors or patients (Figure 1).

In 1998, Kononen et al. (1) published the first report using TMAs for high-throughput molecular profiling of cancers; at the same time, the report highlighted some of the technique's great advantages. First and foremost, the high-throughput nature of the approach means that studies on a large number of patient tissues can be assembled on just a handful of TMA blocks. This can substantially reduce the cost of consumables, and can help prevent the depletion of tissue that should remain in the diagnostic archive for eventual re-evaluation. Staining procedures can be carried out simultaneously across different cores, eliminating experimental variability. Dozens of different biomarkers can be applied to serial sections of the TMA, ensuring its viability as a longstanding research tool for many study groups. Attributes like these mean that TMAs have become a mainstay in translational research.

What is the problem?

On the other hand, conventional tissue microarraying has always presented one major hurdle: the inaccuracy of punching. Typically, a region of interest is marked on the H&E slide, indicating that a core should be punched out from the encircled area. But when the tissues are cored out, there's no physical alignment of the block and slide. This imposes a major limitation on biomarker research, because some specific targets exist only in certain histological areas and aren't captured adequately – or at all. There have been no major developments in tissue microarraying since its inception, but now, with the substantial strides made in digital pathology, the technique is primed to evolve. At the Institute of Pathology of the University of Bern, TMAs are designed based on a next generation tissue microarray (ngTMA) approach that relies on a process we call "VDA²": visualization, digitization, automation and analysis.

Visualization

With ngTMA, our vision is to answer targeted research questions by optimally visualizing and evaluating biomarkers in tissue. Each ngTMA is designed with a specific purpose in mind – so each one is unique and allows planning for special considerations like tumor heterogeneity. Determining which specific histological interfaces are relevant for the study will help to determine the core size that should be used for punching the regions selected, as well as the number of cores per tissue sample. The number of biomarkers to be investigated will determine how many sections need to be cut and provide information on how many ngTMAs should be constructed. Consideration is also given to power and downstream statistical analysis – meaning that, no matter the experiment, we can design ngTMAs that efficiently deliver the necessary results.

Digitization

The integration of digital pathology into the TMA workflow opens new dimensions for biomarker research. Conventional (H&E or immunostained) histological slides can now be scanned and then annotated using a digital slide viewer. We can paste any number of TMA annotations, in various colors and sizes (0.6, 1.0, 1.5 or 2.0 mm in diameter), onto the scanned slide and move them to our histological region of interest (Figure 2). We can then align an image of each donor block to the digital scan containing these annotations and use their coordinates for precise coring and ngTMA construction. Annotations can be placed in any area of the digital slide, and the precision of the alignment

At a Glance

- *Tissue microarrays (TMAs) are increasingly used for biomarker evaluation and validation*
- *Conventional TMAs lack accuracy in punching, meaning that some areas of interest are captured inadequately or not at all*
- *Next generation TMAs (ngTMAs) are digitally scanned, annotated and cored, resulting in more precise alignment and better coring to optimize research*
- *Now that we've achieved precision coring, the next step for ngTMAs is to multiplex antibodies and improve image analysis*

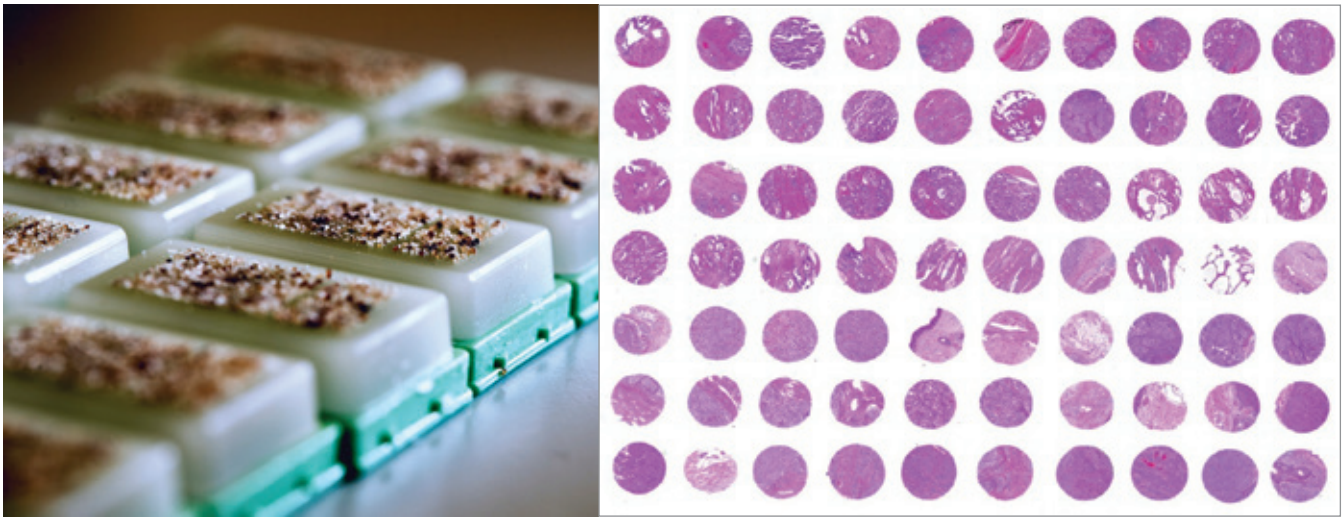

Figure 1. Left: a set of ngTMA blocks just after construction. Right: part of a large ngTMA with H&E staining.

between block and slide guarantees that the marked spot is cored out correctly.

Automation

We can core blocks using an automated tissue microarrayer. As such, the speed and convenience with which we can generate TMAs is far beyond that of conventional techniques. For example, three TMA blocks containing 475 spots each would take approximately 84 hours to construct using a homemade or semi-automated device, but under three hours with an automated arrayer. The automated approach also minimizes loss of tissue cores and maps each core to a particular position within the TMA grid, so the risk of error or spot confusion is negligible. Actual images of the cored donor blocks and their corresponding annotations can also be taken, ensuring traceability of each TMA spot back to the original block for quality assurance.

Analysis

Combined with digital image analysis, ngTMA is a powerful tool. It allows researchers to generate objective and reproducible data to help fast-track biomarker validation, and it can be

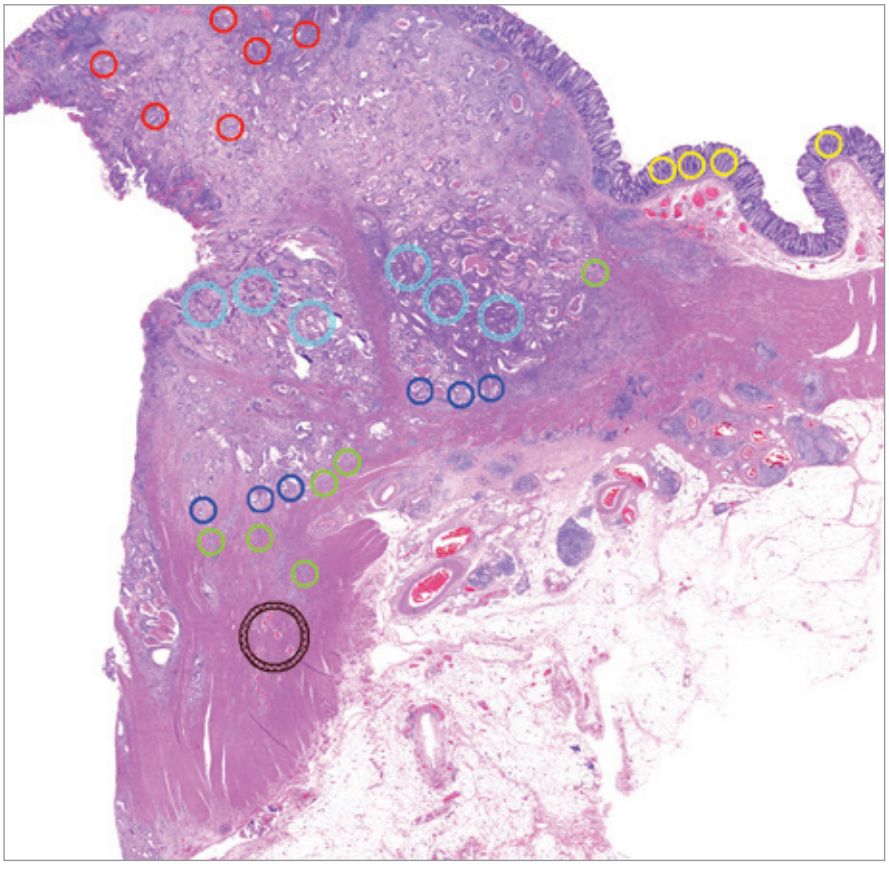

Figure 2. Scan of an H&E stain from a colorectal cancer. Digital annotations of different sizes and colors indicate the regions of interest. The next step in the ngTMA process is alignment of the annotated scan with a donor block image. Annotated regions are then cored and an ngTMA can be constructed.

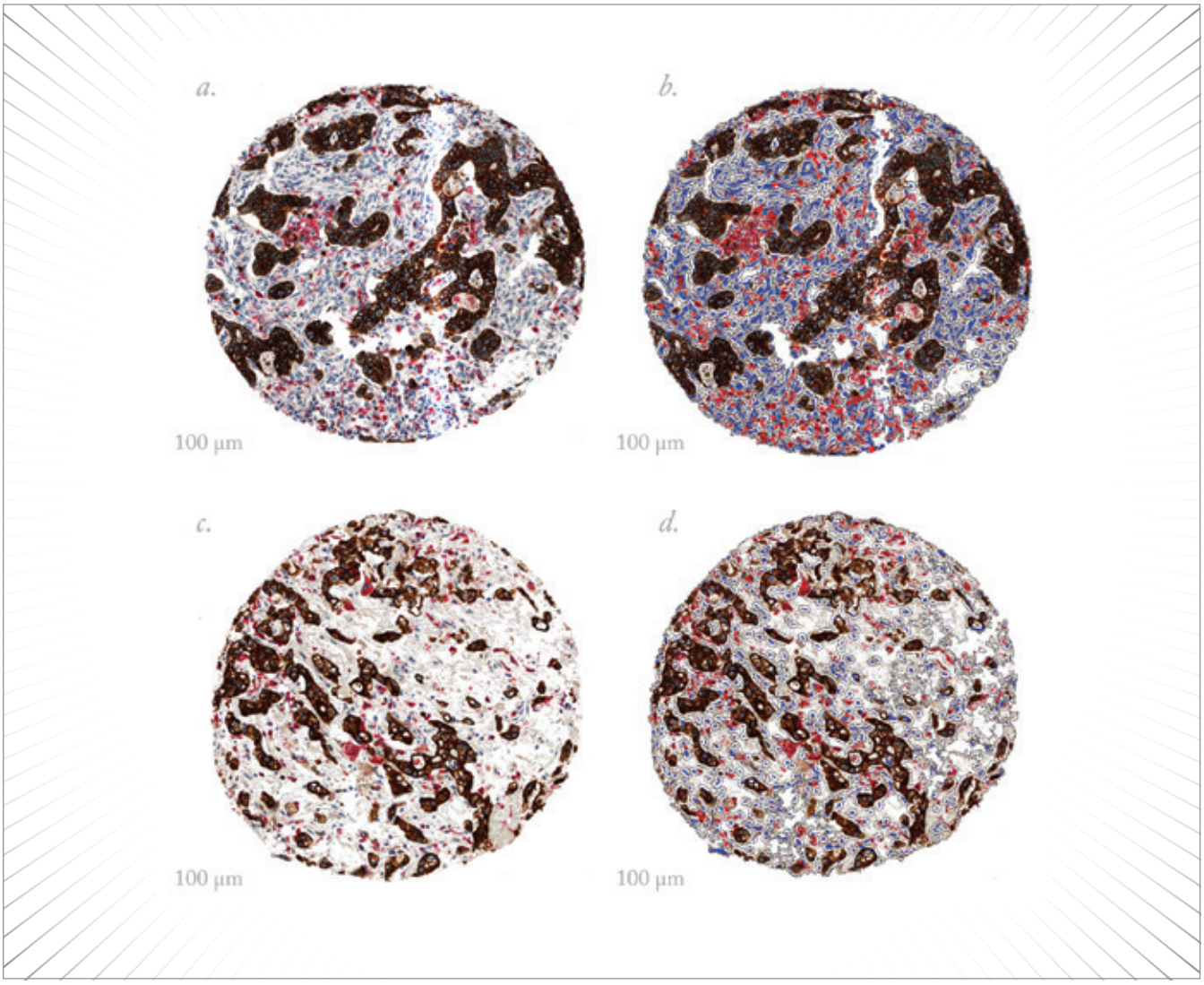

Figure 3. Digital image analysis of an ngTMA that has undergone double immunohistochemistry for pan-cytokeratin (brown) and CD68 (red). This image shows CD68+ macrophages at the invasion front of a colorectal cancer.

used as an investigative tool to further unmask the biology of cancers.

An excellent example to showcase the power of the combined ngTMA/digital image analysis approach is the tumor microenvironment of colorectal cancers. The tumor microenvironment is emerging as a highly relevant histological area for the identification of novel prognostic and predictive biomarkers. Not only T cells, but also macrophages, fibroblasts, and other stromal cells are known to play pro- or anti-tumor roles associated with better or worse patient outcomes (2,3) (Figure 3). In fact, depending on the context, some cell types may have both pro- and anti-tumor functions – meaning that the number of biomarkers required to help tweeze out these contributions and subclassify cell populations is growing. The region at the tumor front where the most invasive cells meet the tumor stroma holds a wealth of potential information we still need to measure, quantify and ultimately use. Recently, the presence of tumor budding was shown to be an adverse prognostic factor, but also that the balance between attacker (tumor buds) and defender (CD8+ T cells) may have a more relevant clinical impact than either feature alone (4) (Figure 4). How can we get the

most information out of these clearly relevant findings?

ngTMAs of the tumor microenvironment, combined with digital analysis, will help unravel the contributions of different cell types to tumor progression, metastasis and ultimately clinical outcome. Pattern recognition can be used to identify different structures at the invasion front. We can even measure staining intensities and set thresholds for positive staining. Three-dimensional reconstruction of the invasion front will help determine the spatial distribution between different cells and structures and even identify apparent contact between cell types. These features supplement "standard" image analysis like quantifying the number of positive cells within a region of interest or separating by color, size, shape or other morphological or cytological features.

"ngTMA is an opportunity to take biomarker research to a new level in clinical and translational settings."

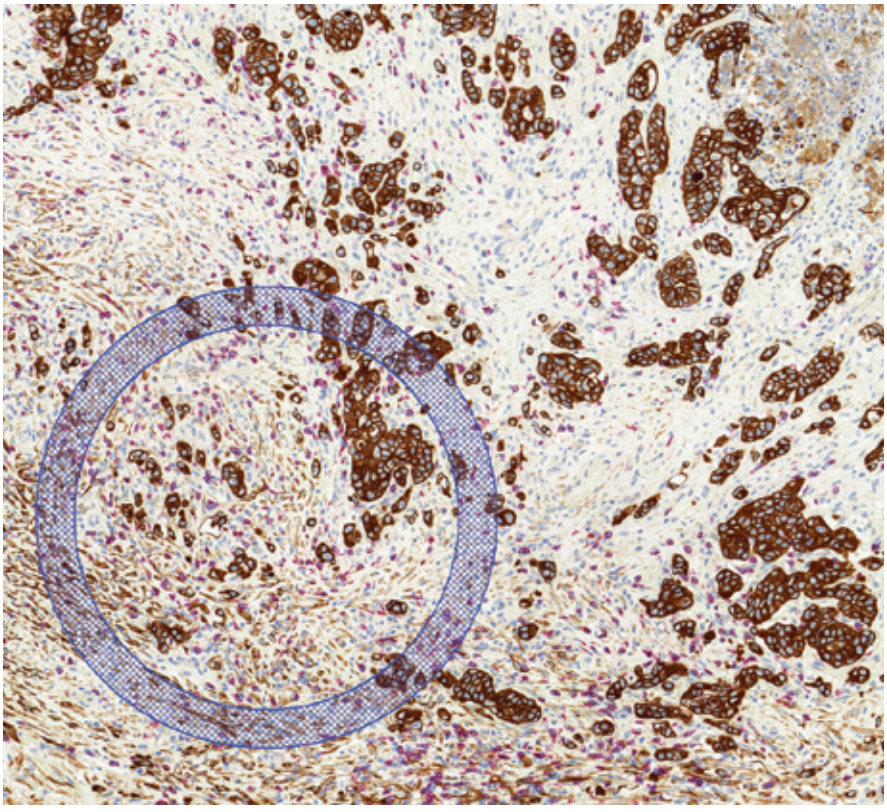

Figure 4. Scan with ngTMA annotation of a colorectal cancer, highlighting a dense area of tumor budding (pan-cytokeratin; brown) and surrounding CD8+ lymphocytes (red). The annotated region can be cored out and included into an ngTMA.

What does the future of ngTMA hold?

Areas like the tumor microenvironment highlight our need for multiplexing. Although we can now capture precise histological areas using ngTMA, we are still limited by our ability to multiplex various antibodies or probes, especially using chromogenic methods. Not only should we be able to do this in the lab, but image analysis should ideally allow us to separate out these components from actual immunostained slides. It's true that this kind of in-depth work will send the amount of data we produce skyrocketing – so it will become increasingly important to work out the best ways of streamlining our analyses and sharing them with other researchers.

ngTMA is an opportunity to take biomarker research to a new level in clinical and translational settings. Because it's a method that combines histopathological expertise with targeted research questions, the latest digital pathology technology, and automated arraying, ngTMA is a way of achieving better characterization and validation of biomarkers – and one I expect will continue to grow in popularity.

Inti Zlobec is Head of the Translational Research Unit (TRU) at the Institute of Pathology, University of Bern, Switzerland.

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2. *C Isella et al., "Stromal contribution to the colorectal cancer transcriptome", Nat Genet, 47, 312–319 (2015). PMID: 25706627.*
3. *J Galon et al., "Type, density, and location of immune cells within human colorectal tumors predict clinical outcome", Science, 313, 1960–1964 (2006). PMID: 17008531.*
4. *A Lugli et al., "CD8+ lymphocytes/tumour-budding index: an independent prognostic factor representing a 'pro-/anti-tumour' approach to tumour host interaction in colorectal cancer", Br J Cancer, 101, 1382–1392 (2009). PMID: 19755986.*

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A Kryptonite for Pathogens

Could antibiotics that target virulence factors be the key to defeating multi-drug resistant "bugs of steel?"

By Timothy Wencewicz

For any pathologist working with infectious diseases, "superbugs" are a well-known and ever-increasing threat. These pathogens have developed resistance to multiple antibiotics – in some cases, to every antibiotic drug we possess. Naturally, this kind of resistance is a significant threat to patients, especially in cases of immunosuppression or frequent visits to doctors or hospitals. But how can we defeat it? Antibiotic development is slow, and resistance evolution is fast. Worryingly, it takes only a year or two after a new drug is introduced – sometimes less – for resistant strains to emerge in the clinic. So how can we fight an enemy that regenerates so rapidly? One answer is by targeting virulence factors, rather than targeting the growth or survival mechanisms of the pathogens themselves.

Credit: Shangwen Luo/University of Illinois at Chicago

Timothy Wencewicz (left) and third-year graduate student Justin Shapiro (right) hold a molecular model of pre-acinetobactin, a siderophore virulence factor produced by pathogenic strains of *Acinetobacter baumannii*.

At a Glance

- *Battling antibiotic resistance is increasingly difficult as more and more pathogens evolve resistance to traditional antibiotics*
- *One way of defeating this is to target virulence factors, rather than whole pathogens, sidestepping the evolution of resistance*
- *Virulence factors affect a bacterium's ability to infect or damage a host, rather than its ability to survive*
- *Targeting such pathways may yield non-traditional antibiotics that are more powerful and versatile than our current antimicrobials*

Targeted treatments

There are two main types of "magic bullet" antibiotics that have dominated the clinic for the past 75 years: bacteriostatic and bactericidal antibiotics. Both fit the general definition of an antibiotic; a substance that halts the growth of bacteria or kills them outright. Bacteriostatic antibiotics stop bacteria from growing, which prevents the infection from spreading but still relies on the immune system to clear the pathogen. Bactericidal antibiotics kill bacteria outright and actually decrease the bacterial load associated with an infection, without needing direct assistance from the immune system. But these treatments come with a clear downside – both types of agent put strong selective pressure on a bacterial population, creating an environment that allows resistant bacteria to overcome their normally susceptible companions.

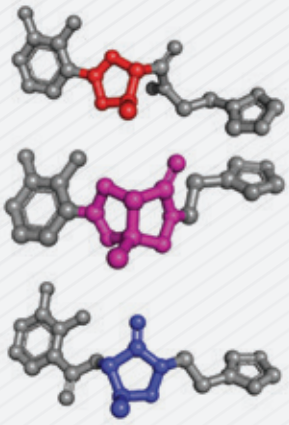
The entire antibiotic industry was built on the discovery and development of these traditional types of antibiotics. Hospitals' infectious disease departments were built around prescribing these types of antibiotics. Now, several factors are challenging the global antibiotic infrastructure: the rapid rise in antibiotic resistance, the decline in antibiotic discovery, and

Acinetobacter baumannii

A. baumannii is a Gram-negative coccobacillus that acts as an opportunistic pathogen. Like other *Acinetobacter* species, it lacks cytochrome c oxidases, but possesses a number of virulence factors and determinants, including pathogenicity islands, beta lactamases, protective polysaccharide capsules, efflux pumps, and adhesion proteins. As a result, *A. baumannii* infections are often multi-drug resistant, and treatment frequently relies on polymixins – drugs so nephrotoxic that they're considered a last resort in most patients.

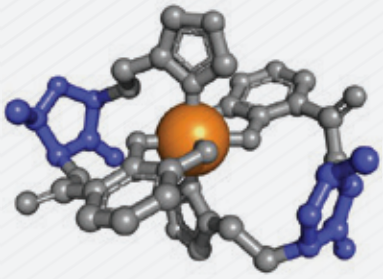
Timothy Wencewicz and his team focus their research on the ability of *A. baumannii* to secrete siderophores that enhance the organism's ability to scavenge iron from its host. Pre-acinetobactin is a siderophore most effective at acidic pH (<6.0). When the pH is higher, the siderophore undergoes a pH-triggered isomerization to become acinetobactin, which is most effective at basic pH (>7.0). It's a "two-for-one" deal for the bacterium, ensuring that it retains its ability to sequester iron regardless of the pH of the host environment.

It's possible that this strategy occurs in other bacteria as well; pre-acinetobactin is not the only siderophore to isomerize. But understanding how these molecules function may pave the way to targeting them with antibiotics. Of course, this is just the first step in a long journey from molecular understanding to clinical applications. But in a world where drug resistance is a looming threat and *A. baumannii* is a challenging opponent, a better understanding of what we face may be the key that eventually unlocks the ability to defend ourselves against it.



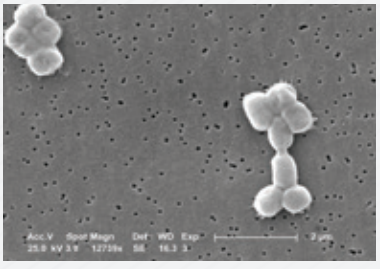
Credit: Tim Wencewicz.

The siderophore pre-acinetobactin (top) isomerizing to acinetobactin (bottom)



Credit: Tim Wencewicz.

Acinetobactin, pictured with a molecule of scavenged iron (orange).



Credit: Janice Carr.

Scanning electron micrograph of *Acinetobacter baumannii*.

the depletion of effective prescription antibiotics. In the past decade, Big Pharma and federal funding institutions have placed significant emphasis on finding new, non-traditional antibiotics that apply less selective pressure (and are thus less likely to prompt the evolution of resistance) – a tall order considering resistance has been happening for hundreds of millions of years and will continue to happen as long as life is present on Earth. Nonetheless, this is when the concept of antivirulence antibiotic strategies truly began to gain traction.

Answers in antivirulence

Antivirulence antibiotics target bacterial pathways that are specific for pathogenesis or host invasion. These pathways aren't needed for the day-to-day life processes of bacteria when growing in a test tube; they're only activated when the pathogen attempts to establish itself inside a host. So in a host environment, antivirulence antibiotics behave like bacteriostatics – but in a "test tube" situation, they have no antibiotic effects at all. As good as that sounds, it gets straight to the heart of why we don't currently have antivirulence antibiotics in the clinic. The platform that companies use to discover traditional antibiotics relies on screening large libraries of molecules in assays that test for direct inhibition of bacterial growth in a test tube. It's far more challenging to develop assay conditions that mimic the human infection environment – but that's what we would need to find new antivirulence antibiotic lead compounds.

It also doesn't help that, inherent in the screening assay design, there's a lack of understanding of the fundamental pathways that drive virulence. Personally, I support revamping the entire antibiotic pipeline, not only for non-traditional antibiotics like antivirulence compounds, but also for traditional "magic bullets." Both will have a place in the long-term

management of the global antibiotics market, and academic researchers like me can contribute by exploring new biological pathways, validating new targets and synthesizing new chemical structures that address as-yet unmet needs. My lab, for instance, studies iron acquisition pathways in bacteria. Because the battle for scarce iron can determine the overall course of an infection, many researchers consider these pathways the Achilles heel of pathogens.

"Our hope is that diagnostic science will keep up with the development of non-traditional antibiotics, so that we can use these tools in tandem against the growing threat of superbugs."

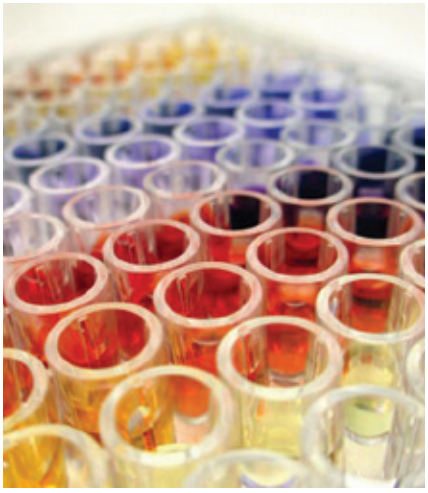
Our research focuses on working out the fine details of how bacterial virulence systems function at the molecular level. We plan to use the natural molecules involved with bacterial iron scavenging from the host as starting points for building molecules that compete with and ultimately block the natural pathway. We are currently focused on the multi-drug resistant Gram-negative bacterial pathogen *Acinetobacter baumannii*, which uses a cocktail of four siderophore (iron-carrying) molecules for scavenging iron in the human host. Our recent research (1) has revealed the pH-triggered mechanism these bacteria use for iron acquisition – but more than that, it highlights our approach to understanding the molecular mechanisms of virulence. Hopefully, this kind of research lays the groundwork for establishing predictive antivirulence assays, so that one day we'll be able to screen for "kryptonite" molecules that turn super-pathogens into ordinary Clark Kent bugs.

Tailoring treatments

In the human body, antivirulence antibiotics behave like bacteriostatics – with one significant difference. Bacteriostatics halt pathogen growth by inhibiting a pathway required for primary life processes – but this creates more selective pressure, inducing resistance. Antivirulence antibiotics halt growth by inhibiting pathways required for invasion of host tissue or evasion of the immune system. Those aren't primary life processes outside the host environment, so they don't select for resistance in the same way.

The exact mechanism by which antivirulence antibiotics and the immune system work together to clear infection is difficult to predict and highly dependent on the antivirulence strategy. For example, we can block the production of a virulence factor that allows the pathogen to evade the immune system; then, the pathogen is no longer able to enter "stealth mode" and the immune system can successfully detect and clear it. In my laboratory, we block iron acquisition pathways and essentially "starve out" the pathogen, holding it hostage in a non-virulent state and buying time for the immune system to clear it naturally.

All human bacterial pathogens are susceptible to antivirulence antibiotics, but not all in the same way. Virulence pathways are unique to each pathogen because they're tailored to enable pathogenesis in a specific tissue and environment. Although some strategies are more widely applicable than others, the general thought in the field is that antivirulence antibiotics should be tailored to a target pathogen. That's both a scientific and an economic challenge, because it narrows the market for the drug and demands rapid and accurate diagnosis of the pathogen in question. The reason we so often use broad-spectrum antibiotics is because we lack good diagnostic tools for rapid bacterial identification. The opposite face of that argument is that narrow-spectrum antivirulence antibiotics are attractive from a resistance standpoint, because the selectivity of the target pathway protects other bacteria – including the healthy human microbiome. Our hope is that diagnostic science will keep up with the development of non-traditional antibiotics, so that we can use these tools in tandem against the growing threat of superbugs.



Credit: Tyler Morse/Washington University in St. Louis.

An assay using a range of siderophore concentrations.

Timothy Wencewicz is an assistant professor in the Department of Chemistry at Washington University in St. Louis, USA.

Reference

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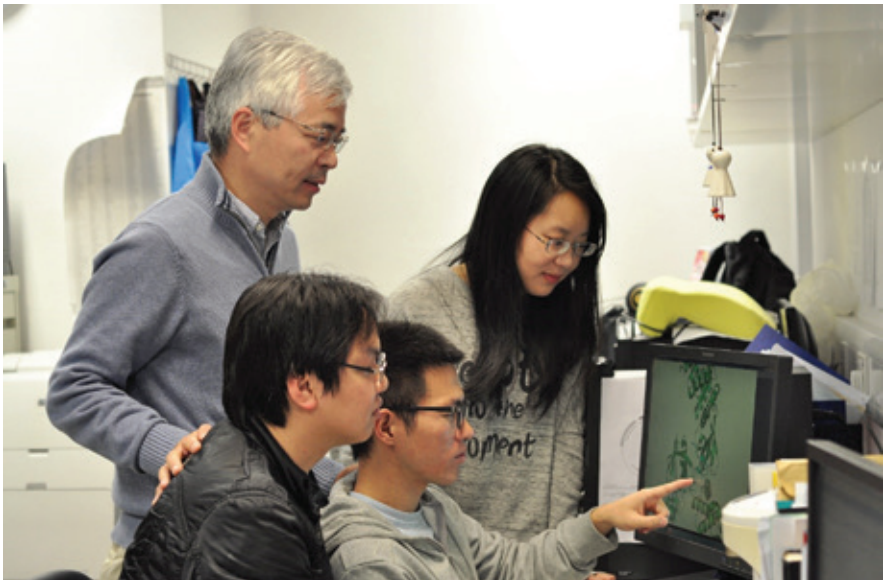
Listen to Your Gut

Recent studies reveal that the organizational protein complexes in gut microvilli closely resemble those of ear stereocilia – so what does this mean for genetic disorders of either?

By Michael Schubert



Members of the Zhang laboratory (from left to right: Mingjie Zhang, Jianchao Li, Yunyun He, Qing Lu).

When considering the function of the ear, the gut doesn't automatically come to mind as a research model. After all, what could two such different tissues have in common? Unexpectedly, the answer lies in some of the most vital functional components of each organ – namely, the gut microvilli and the stereocilia of the ear. These two structures behave in very different ways; while the job of the microvilli is to add surface area to the intestinal epithelium and function in nutrient absorption, stereocilia are responsible for hearing and balance. But what they share is the protein complex that organizes them. Although ear and gut use different complexes, each protein subunit in one has an exact counterpart in the other – one of which even derives from the same gene in both tissues. And as if to mirror this duplication of complexes, two groups published similar findings at the same time in the journal Developmental Cell (1,2). We spoke to Scott Crawley from Vanderbilt University and Jianchao Li from Hong Kong University of Science and Technology to find out more about the organizational proteins of both gut and ear.

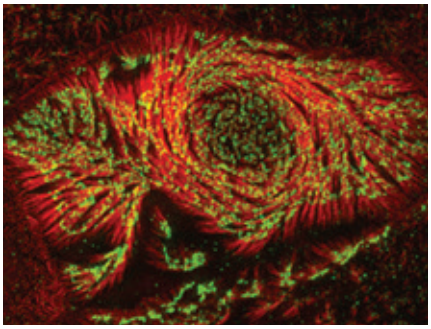
At a Glance

- *The organizational protein complexes of gut microvilli and ear stereocilia are very alike, with each protein subunit of one having an exact counterpart in the other*
- *Both complexes possess two cadherins, one myosin motor protein, and two scaffolding molecules, which together link the actin-based apical protrusions at their distal tips*
- *Damage to the ear complex can lead to Usher syndrome, the most common cause of congenital deaf-blindness – but it's not yet understood why similar damage to the gut complex doesn't cause an equally severe phenotype*
- *Better understanding of these complexes may lead to easier diagnosis of genetic disease, or may help us discover useful information for its treatment*

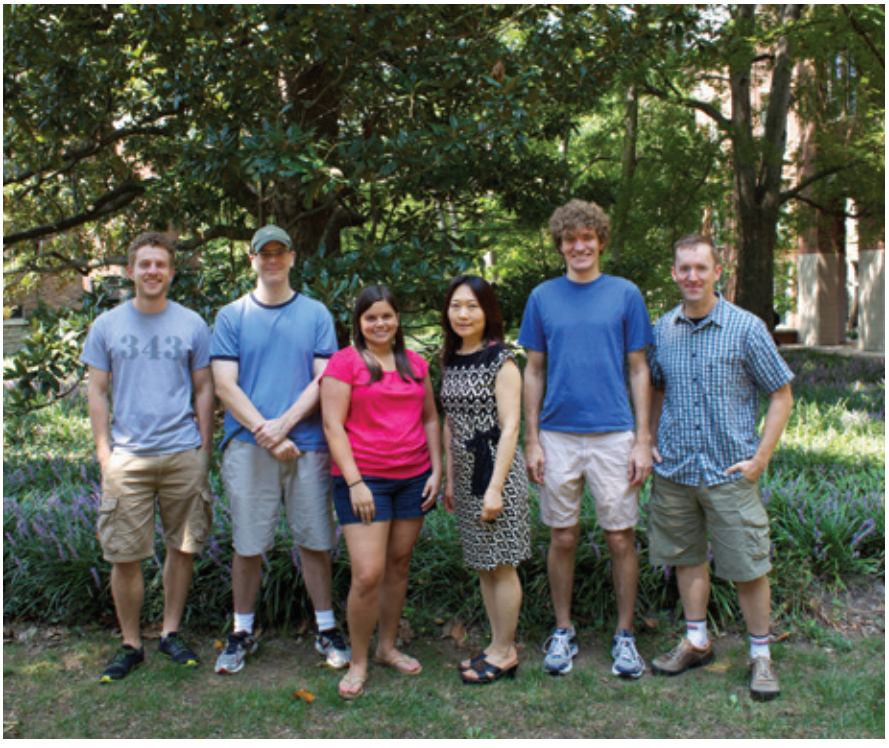
Why study the gut microvilli?

Jianchao Li: We were initially interested in inherited deafness, especially in Usher syndrome, a rare genetic disorder caused by genetic mutations that lead to impairments in both vision and hearing. Previous studies by scientists in the gut field suggested that there might be some kind of linkage between gut and ear, given that the microscopic structures are quite similar — the stereocilia in the ear hair cells and the microvilli in the gut epithelia. The more we looked into these structures, the more we realized that this was true.

Scott Crawley: The ultrastructure of gut microvilli has long been a fascinating research subject. When scientists first started looking at them, they named them the "brush border" – a very apt term, because they collectively resemble a scrub brush. Although there are countless interesting aspects of the brush border, I think the thing that drew me to study this aspect of biology is the amazing order exhibited by the system. How exactly an intestinal epithelial cell creates upwards of 1,000 finger-like membrane protrusions on its surface (the "bristles" of the brush), all of which exhibit uniform length, is a remarkable feat of biological engineering. We wanted to know what proteins contribute to this process.



An immunofluorescence image of the gut microvilli (green: myosin-7B, red: F-actin cytoskeleton; yellow: colocalization).



Members of the Tyska laboratory (from left to right: David Shifrin, Scott Crawley, Meredith Weck, Suli Mao, Nathan Grega-Larson, Matthew Tyska).

Can you describe the protein complex that organizes the microvilli?

JL: Gut microvilli are the finger-like protrusions that line the intestines. The backbones of these protrusions are made of actin proteins. What our research has revealed is how the key protein components interact with each other to assemble the microvilli – and, along with that, the fact that these protein components are strikingly similar to those essential for the assembly of stereocilia (hair-like mechanosensory organelles) in the inner ear.

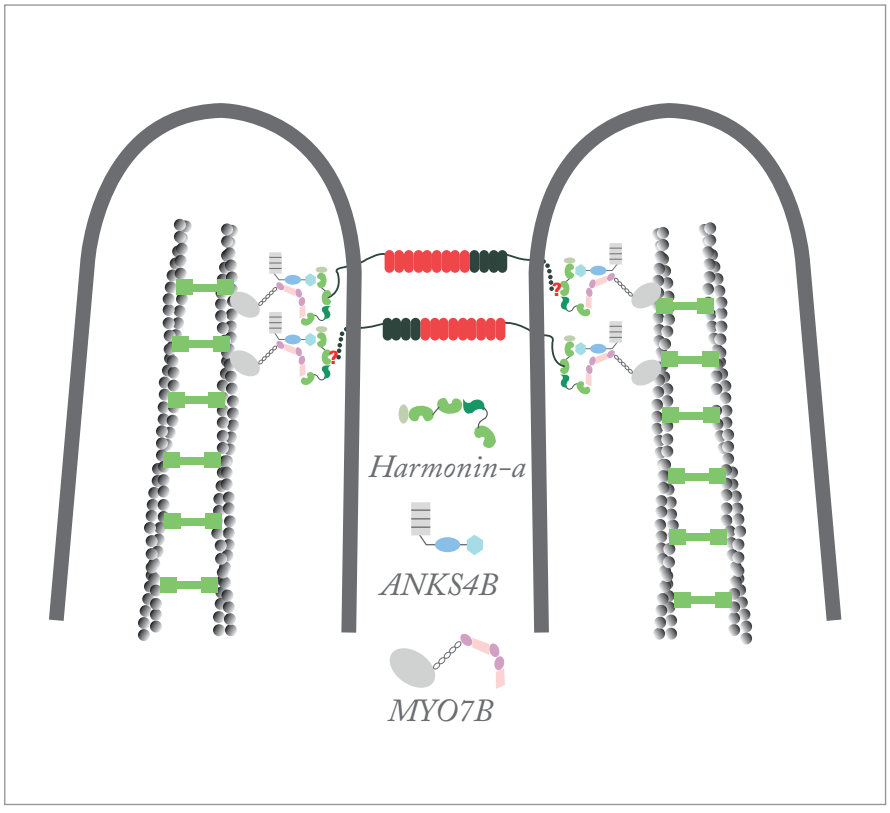
SC: We found that, during the formation of the brush border, microvilli are physically connected to one another through small, thread-like links at their distal tips. We discovered that these thread-like links are composed of a pair of cadherin adhesion molecules – a class of proteins that essentially act like "molecular Velcro." Cadherins glue opposing membrane surfaces together, and that's exactly what they do in the brush border: they glue neighboring microvilli together. Specifically, we found that the thread-like links are made of protocadherin-24 and mucin-like protocadherin. Both cadherins localize to the distal tips of microvilli through interactions with a myosin motor protein (myosin-7B) and two scaffolding molecules (harmonin-a and ANKS4B). We termed this complex the intermicrovillar adhesion complex (IMAC).

Interestingly, the IMAC is very similar to an adhesion complex that connects the stereocilia of the inner ear. Each protein component of the IMAC has a functional counterpart in the inner ear adhesion complex (known as the Usher complex, because genetic defects in its components cause Type I Usher syndrome, the most common form of deaf-blindness). All of the proteins in these two complexes come from different genes, with the exception of harmonin, which uses different splice variants of the same gene. Interestingly, Usher syndrome patients with defects in the harmonin gene also suffer from intestinal disease. So, although the gut and the inner ear are two completely different organs (with completely different functions!), their epithelial cells use a common adhesion mechanism to remodel their apical surfaces just after the cells are born.

What are the implications of this discovery?

JL: This information provides a starting point for those who are studying gut microvilli to look for functional alterations that may lead to disease. Considering systems with similar structures (like the gut, the ear and the kidney), it's possible that changes to a single gene might cause problems in several of these systems.

SC: I think studying Usher syndrome has been particularly challenging for researchers because there's no cell culture model system for the inner ear epithelium. When researchers want to study whether and how a particular mutation causes Usher syndrome, they typically have to make a genetically modified mouse to see how the mutation affects the formation of stereocilia – an expensive and time-consuming process. In contrast, we have excellent cell culture model options for the intestine.



A diagram of the protein organizational complexes in the intestinal microvilli.

Now that we know the IMAC and the Usher complexes are so highly homologous, we can use the IMAC as a substitute to investigate how Usher syndrome mutations cause disease. We can compare the two complexes, make the equivalent Usher syndrome mutation in the IMAC component, and watch how it affects brush border formation. I think this will give us an unprecedented opportunity to understand the molecular pathology of Usher syndrome, and it may just bring us one step closer to finding effective treatments.

How will this affect the diagnosis and treatment of intestinal disorders?

JL: In contrast to the corresponding genes in Usher syndrome, very little human genomic data so far has linked the IMAC genes to intestinal disorders. Our work on elucidating the protein-based organization of the brush border may help predict which mutations are likely to alter function, bringing genetic diagnoses into clinicians' sights. Digestive disorders are likely to be polygenic, so one genetic mutation alone may not lead directly to disorders – but changes to even a single gene can make the gut system more vulnerable.

SC: We know that Usher syndrome patients with defects in the harmonin gene can suffer from intestinal disease along with the usual deaf-blindness. This leads us to believe that genetic defects in the other IMAC components might cause similar intestinal disease. Knowing that IMAC genes could potentially cause disease should allow us to rapidly screen patients with undiagnosed intestinal illness for potential mutations in their IMAC components. That would give us a starting point to understanding why these patients are sick – and hopefully, in the future, lead to solutions.

What are the next steps for your research?

JL: Another puzzle left to solve is why a single mutation can cause a disease as debilitating as Usher syndrome, while corresponding mutations in gut microvilli don't lead to noticeable symptoms. If the gut system is older in evolutionary time and the digestive function more crucial for life, it could be that the gut system has developed more robust backup systems, or that the symptoms are associated with additional genetic factors we have yet to discover.

SC: I'm interested in trying to understand how the IMAC and Usher complexes assemble in epithelial cells. Our latest research suggests that assembly of these complexes inside the cell is a highly regulated process. By obtaining a better understanding of how they assemble, I think we will garner a lot of insight into how disease-causing mutations disrupt the normal function of these adhesion complexes.

Scott Crawley is a postdoctoral trainee in the Matthew Tyska laboratory of the Department of Cell and Developmental Biology, Vanderbilt University School of Medicine, Nashville, USA.

Jianchao Li is a postdoctoral researcher in the Mingjie Zhang laboratory at Hong Kong University of Science and Technology, Clear Water Bay, Kowloon, Hong Kong.

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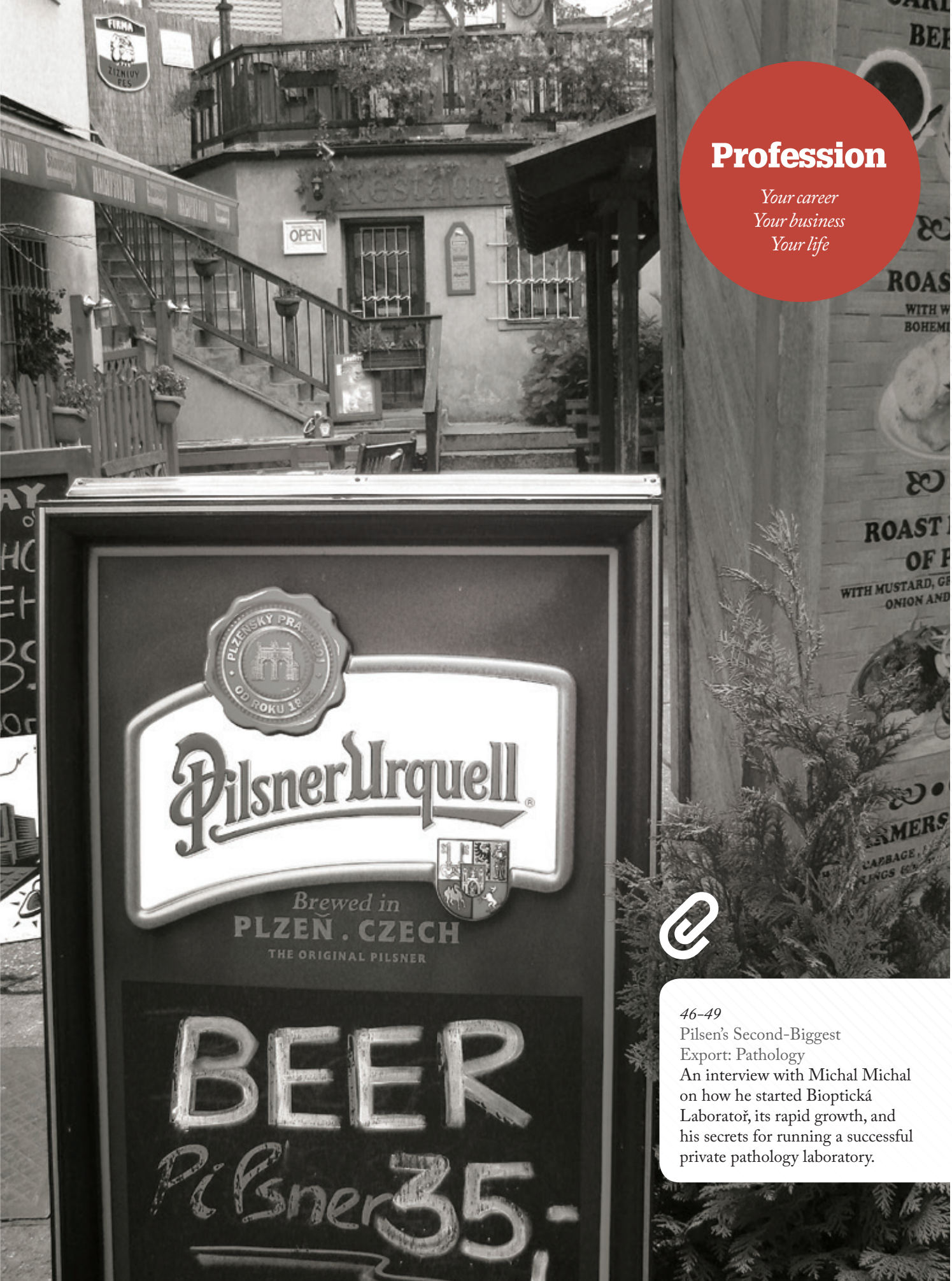

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Pilsen's Second-Biggest Export: Pathology

From plastic beer case furniture to the largest pathology laboratory in Eastern Europe

Ivan Damjanov interviews Michal Michal

Pilsen (or Plzeň to its inhabitants) is a city with many claims to fame. The fourth largest city in the Czech Republic, it served as the cultural capital of Europe in 2015. But of course, it's best known for Pilsner beer, which was created many years ago in one of the breweries of that medieval town. The Plzeň of today still brews beer in enormous quantities, but now there's something even more noteworthy about it – it's the home of Bioptická Laboratoř (BL), the biggest pathology laboratory in the Czech Republic. I recently had the opportunity to ask Michal Michal, the head of this enterprise, a few questions about it. You might be interested in finding out how he became the richest pathologist in the Czech Republic!

At a Glance

- *Over the past 23 years, Bioptická Laboratoř has grown from one microscope balanced on a cardboard box to the largest private pathology laboratory in Eastern Europe*
- *It was a matter of "right place, right time" and a true entrepreneurial attitude for Michal Michal, who heads the laboratory and obtained its first private license in 1993*
- *Bioptická Laboratoř now provides pathology services to most of the Czech Republic and a significant portion of the surrounding countries*
- *The lab's infrastructure is forever expanding – from multi-head microscopes and electronic records to housing and car care for its staff!*

Let's start with some history. How did you expand Bioptická Laboratoř from a small, rented flat into the largest laboratory in Eastern Europe?

Bioptická Laboratoř was established in 1993. The name translates to "Biopsy Laboratory" in English, a reflection on the fact that, at the very beginning, we only did biopsies – cytology investigations began four years later, and genetics six. When we started, we didn't have any money. There was one small microscope shared between three pathologists, and instead of a table and chairs, we used empty plastic beer cases arranged around the box from a computer monitor. We rented a small flat in a house in the center of Plzeň that belonged to my brother, but when his small business went bankrupt, we faced a dilemma: whether to buy the house so that my brother could repay his debts, or simply move out. In the end, we took out a loan from the bank for the price of the house – CZK3,000,000 (about US$100,000). At the time, that was a lot of money; the net salary of a pathologist was only about CZK3,000 per month. The same house would cost 10 times as much today, but luckily, we started early. Now, we've expanded to six neighboring buildings in a block in the middle of town – including an old cinema called Eden, which used to be the biggest cinema in communist Czechoslovakia. We have entirely rebuilt it and brought our laboratory space up to 8,500 m^2.

I am happy to confirm the rumors circulating about our lab's size! There's no question that Bioptická Laboratoř is by far the biggest private laboratory in Eastern Europe. We process over 160,000 biopsies and 800,000 cytological specimens a year, as well as 10,000 consultation biopsies from around the world and 80,000 immunohistochemical stainings. Next year, we expect to process biopsies and cytological examinations for over a million patients!

Michal Michal, head of Czech pathology laboratory Bioptická Laboratoř.

What does Bioptická Laboratoř do now?

This year, we've started something new: producing immunohistochemical slides for other pathology departments. We employ about 450 primary antibodies in our immunohistochemical laboratory – more than any other laboratory in the Czech Republic can store, to the point where some hospitals have replaced their immunohistochemical laboratories altogether with our services. Our IT technicians wrote a program that we install on the hospital computers, so that pathologists can just input a patient's number and click to select the tests they want us to conduct. We produce the slides, scan them with a whole slide scanner, and send the virtual slide back. The real slides follow a day later by car.

Fifteen years ago, we expanded our services to include molecular biology. Since then, the demand for it has grown rapidly, especially in recent years. We offer nearly 100 genetic tests necessary

for hematopathology, solid tumors (especially soft tissue neoplasms), germline mutations (especially Lynch syndrome), familial adenomatous polyposis, *BRCA* mutations, Brooke-Spiegler syndrome and many others. The last test we introduced has been in great demand; it detects mutations in the promotor of the telomerase reverse transcriptase gene *TERT*. When found in urine sediment, these mutations are practically specific for urothelial carcinoma of the urinary bladder and upper urinary tract, with a sensitivity of 85 to 87 percent.

"Instead of fighting the societal problem of that corruption, we've chosen to concentrate on pathology, provide a first-class service to the public, and use the money we earn for science."

Figure 1. A map of the Czech Republic showing Bioptická Laboratoř's customers.

How much trouble did you have with issues like permissions, licenses and logistics?

It was not difficult at all to license a private laboratory in 1993. The entire Czech healthcare system belonged to the state, and the Ministry of Health declared that anybody who wanted a private license could ask for one, but had to do it by the end of the year. Our biggest logistical problem was organizing the collection and reading of classical cervical-vaginal Pap smears. When you read 800,000 Pap smears a year, it's hard to avoid mixing up specimens – so we had our technicians develop a unique program using barcodes. We provide two barcodes for each test: one for the slide and one for the accompanying documents. That prevents mix-ups without requiring any identifying information. When our pathologists receive the slides, they scan the barcode to get the patient's gynecological history. After examination, the result is sent electronically both to the gynecologist and directly into the patient's file – and if there are any abnormal findings, the gynecologist receives an automatic text message as well.

Now that we've grown so much larger, we've had to develop ways of providing pathology services to the entire country (Figure 1). As a result, our lab owns 90 cars, employs several professional drivers to collect specimens, and has secretaries who also work as part-time specimen collectors (spending mornings visiting clinical clients and afternoons on secretarial work to break up the monotony). We have a unique pay structure for this kind of work; as secretaries, they are paid for every word they type, and as specimen collectors, they are rewarded based on mileage and number of visits. It works for us and for them because it's a very flexible system that grows when we do.

We are also large enough that we don't need to market our services. Our only advertisements are our publications and the lectures our pathologists give worldwide. We pay our pathologists to give those lectures, and talks aimed at clinicians are better paid than those intended for pathologists. We also have a "price list" for papers, to motivate our pathologists to publish in highly ranked journals – so a paper accepted for publication in, for instance, the American Journal of Surgical Pathology, will earn the pathologist much more money than one published in a journal with a lower impact factor. We also run regular courses for pathologists (see www.patologie.cz for more information) and some quite popular Saturday pathology courses for clinicians.

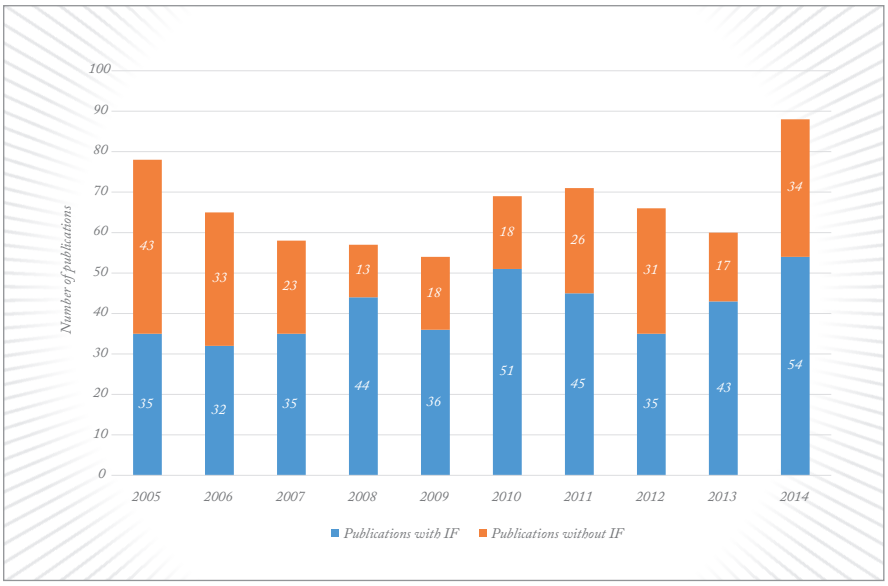

Figure 2. Publications per year by Bioptická Laboratoř. IF: impact factor.

How does the laboratory work as a business?
We don't set our own prices. The costs of pathological and cytological services are regulated by healthcare insurance companies, and the prices of genetic tests are regulated by clinicians. Each clinician has a budget allotted by insurance companies for all laboratory examinations, and if they go over budget, the companies fine them – so they're the main regulators of cost. That said, the prices in our country are consistently much lower than in western countries. For example, insurance companies pay us €6–9 for one immunoslide, whereas in Western Europe the same slide would cost three to six times more.

We have quite a large profit margin on our tests, but we also spend a huge amount of money on things we can't sell – laboratory research and scientific publications. Our pathologists publish between 60 and 80 scientific papers a year (see Figure 2), of which more than half have appeared in high-impact journals. Nowadays, nearly all of those papers include genetic and molecular data, which we pay for without reimbursement. We can't even get government funding because the system of grants is quite corrupt in our country. Instead of fighting the societal problem of that corruption, we've chosen to concentrate on pathology, provide a first-class service to the public, and use the money we earn for science. Our laboratory has been recognized nationally and internationally as a major research center. Our pathologists have been internationally credited for the first descriptions of several tumor types discovered right here in Pilsen (1–8). And of course, we're always expanding; the last few years of income have been spent on renovating the former Eden cinema into a laboratory. We own several blocks of houses in the center of Pilsen and anticipate acquiring even more real estate. As you can see, pathology, business, science and education are all intricately interconnected, and our model of integrating all of these laboratory activities has proven to be a winning formula!

Almost all of the profits that don't go to research are reinvested into the laboratory and its personnel. We don't only buy buildings for the laboratory – we arrange housing for our staff as well, including 15 flats for our pathologists and two apartment houses for other employees. We also pay for services like childcare, car care and household help. In fact, we even have a private mobile phone network that has grown to over 7,000 SIM cards and includes not only our employees, but also our clinician clients and their families. Naturally, all of this costs a lot of money, but we consider it a good investment!

What are some of the most interesting activities at Bioptická Laboratoř?
The 12 geneticists and 10 technicians in our genetic laboratory specialize in the pathology of various syndromes, including many neoplasms and infectious diseases. In that laboratory, we also conduct forensic genetic testing (for instance, paternity testing for legal purposes or genealogical analysis of family lines) and perform 20,000 HPV tests a year. It's our fastest-growing division and I anticipate a lot of interesting work in that area in the future.

We also run an outreach program for pathologists from former communist countries. We invite them, reimburse their travel expenses, and provide accommodation in one of the laboratory's six visitor apartments. We collaborate with pathologists in a number of post-communist countries, working on joint projects with us that they couldn't afford by themselves, and we welcome them frequently for visits. Fortunately, we are well equipped for group projects, with a number of conference rooms and other collaborative facilities – even a multi-head microscope with 31 heads! These facilities have allowed us to host national and international meetings, including a conference of the Arkadi M. Rywlin International Pathology Slide Seminar Club, several on urological or head and neck tumors, and many smaller meetings. And since our 2012 accreditation by the International Committee of Dermatopathology-European Union of Medical Specialists as a Specialty Training Center in Dermatopathology, the number of guests we receive has only grown.

Bioptická Laboratoř staff using one of the laboratory's multi-head microscopes.

What are you most proud of so far – and what do you hope to do next?

I am most proud of the community in our laboratory. We work together as a sort of kibbutz and have great relationships. Our laboratory has 270 employees, and in the 23 years of its existence, only two have ever voluntarily left our employ! It makes me very pleased to think that practically all of the employees who enter our laboratory will stay with us forever.

A major plan of mine is currently in its final stages. We're implementing full electronic communication with all of our clinical clients in over 3,000 locations. Soon, we won't need to use paper documentation for biopsies, cytological examinations or genetic examinations. For that, we've employed four excellent IT technicians, and we're now working on getting permission from all the healthcare insurance companies to allow us to go paperless. Paperwork is mandated by law in our country, so we need to overcome that obstacle – but technically, the project is ready!

I have one other dream: to build a restaurant in one of our houses to serve our employees, visitors and the public. I'm well known for my interest in enology – my private wine collection includes about 13,000 bottles – and our laboratory manager's son is one of the best sommeliers in the Czech Republic, so we often dream of making this a joint project. We've already decided that it will be a French wine restaurant, so now we're just waiting for the right space to be available!

Do you have any secrets that help you beat the competition?

Just one – namely, that almost all of our main managers are women. In our laboratory, women order and men obey. I generally consider women to be more responsible, more focused, and more psychologically stable. We have a joke in our country that when the women take power, they will send all the men to live in the zoo. If that comes to pass, I hope they'll reserve the biggest cage for me.

Ivan Damjanov is Professor of Pathology at the University of Kansas School of Medicine, Kansas City, USA.

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No Regrets

Sitting Down With… Suzy Lishman, President of the Royal College of Pathologists (RCPath) and a consultant cellular pathologist at Peterborough City Hospital, UK.

What is the most challenging objective that you set for yourself to achieve during your time as President of RCPath? What progress has been made toward achieving it?

My key aim was to engage members so that they feel that they're part of the College and that their views are sought and acted on. I've tried to do this by meeting as many members as possible, by keeping them up to date with what the College is doing on their behalf through e-newsletters, our printed Bulletin, social media and our website, and by encouraging them to contact me with their concerns and opinions, which I've represented to government and other policy makers. For example, I have raised concerns about aspects of the new contract for junior doctors that relate to research, less than full-time training and moving to pathology after gaining experience in another specialty. I and others have also campaigned for several years for independent medical examiners to scrutinize all deaths not referred to the coroner. The government has recently announced that medical examiners will be introduced in 2018. The College was also instrumental in having amendments made to the Access to Medical Treatments (Innovation) Bill, which has just received Royal Assent. I have also made appointment to College posts more transparent, with all eligible members being given the opportunity to contribute.

You have placed a big focus on public engagement initiatives. Which have been most effective and why?

Without a doubt, National Pathology Week (NPW), which is held every November. Since it was introduced in 2008, thousands of events have been held, taking pathologists and scientists out of their labs and into their communities. Hundreds of thousands of schoolchildren, students and members of the public have learned about the importance of pathology to their health. We've found that the public are fascinated by what pathologists do and love to learn more about our central role in healthcare. NPW has been so successful that other countries have asked to join us and we now also hold an International Pathology Day every year, focusing on global health issues.

I have worked to strengthen the College's parliamentary and stakeholder contacts since I came into post, meeting regularly with health ministers and establishing relationships with the Health Select Committee. During NPW in 2015, the chair of the health committee, Dr Sarah Wollaston, hosted an exhibition in Parliament organized and run by the College. It was staffed by pathologists to showcase the value of pathology in healthcare. About 50 members of parliament and a dozen peers visited the exhibition, including most members of the health committee and the Secretary of State for Health, Jeremy Hunt.

The Virtual Autopsy has been one of the most engaging and popular activities during NPW. What are your thoughts on the worrying decline of the hospital autopsy?

They are an extremely valuable way to learn more about the progression of disease and its response to treatment. Reversing the trend will be difficult, but I believe it is important to strive to do so. Reform of the coronial autopsy service in England, which is currently being discussed, may help reverse the decline of hospital autopsies by ensuring that there are sufficient trained pathologists and appropriate facilities.

How can pathology be made more attractive to students?

The best way is by increasing students' exposure to the specialty during their training. That is why the College has introduced an undergraduate membership category and co-hosts an annual summer school for students. We have also developed an undergraduate pathology curriculum to encourage medical schools to include core pathology knowledge in all students' training. It is essential for pathologists to act as role models for students, demonstrating the range of career options and highlighting how fulfilling working in pathology can be.

You are the College's second female President; do you feel that you had to work harder than your male colleagues to get to where you are?

It's almost impossible to know how hard other people work – and some people are more efficient than others! I have certainly worked hard, but I doubt if I've worked any harder than my predecessors. I am extremely fortunate to have supportive family and colleagues who have enabled me to take on College roles for many years and who continue to support me while I'm President.

If you could go back and give yourself advice before you embarked on your career, what would you say?

I've loved my career and it's a huge honor to be president of the College so I'm not sure there's anything I'd change. I might tell my younger self that all the hard work would pay off, to make the years of long hours and endless exams a little easier. I would reassure my newly qualified self that pathology really was the career for me and that it would be even more interesting and enjoyable than I hoped when I first applied to enter the specialty. I feel extremely fortunate to have had the support, opportunities, role models and colleagues that I've had over the years. I've also still got many years to work, so my career will be far from over (I hope) when I demit office at the end of 2017. So I may still need that advice from my older self – perhaps ask me again in a decade!

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