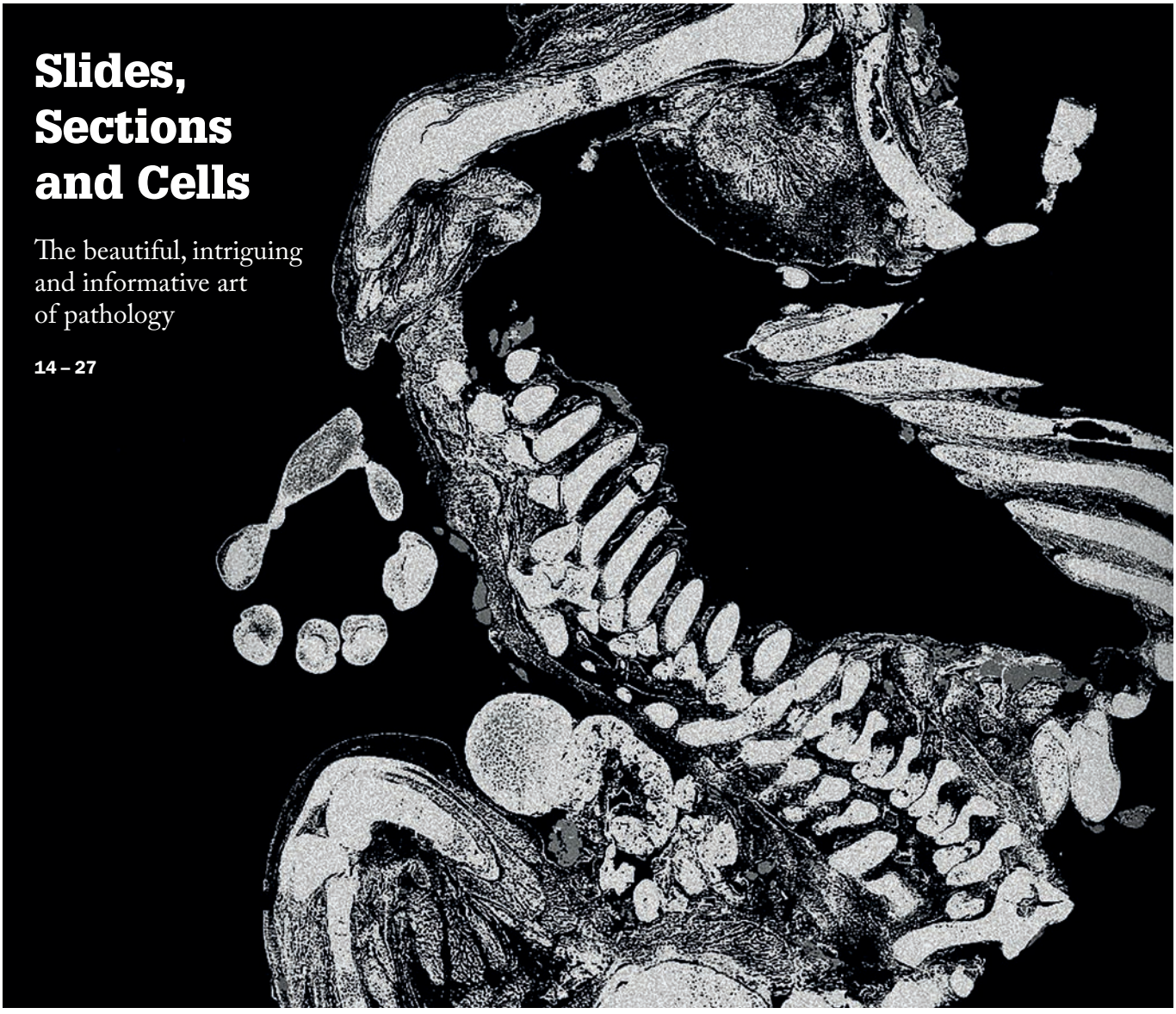
JUNE 2017 # 31

# the Pathologist

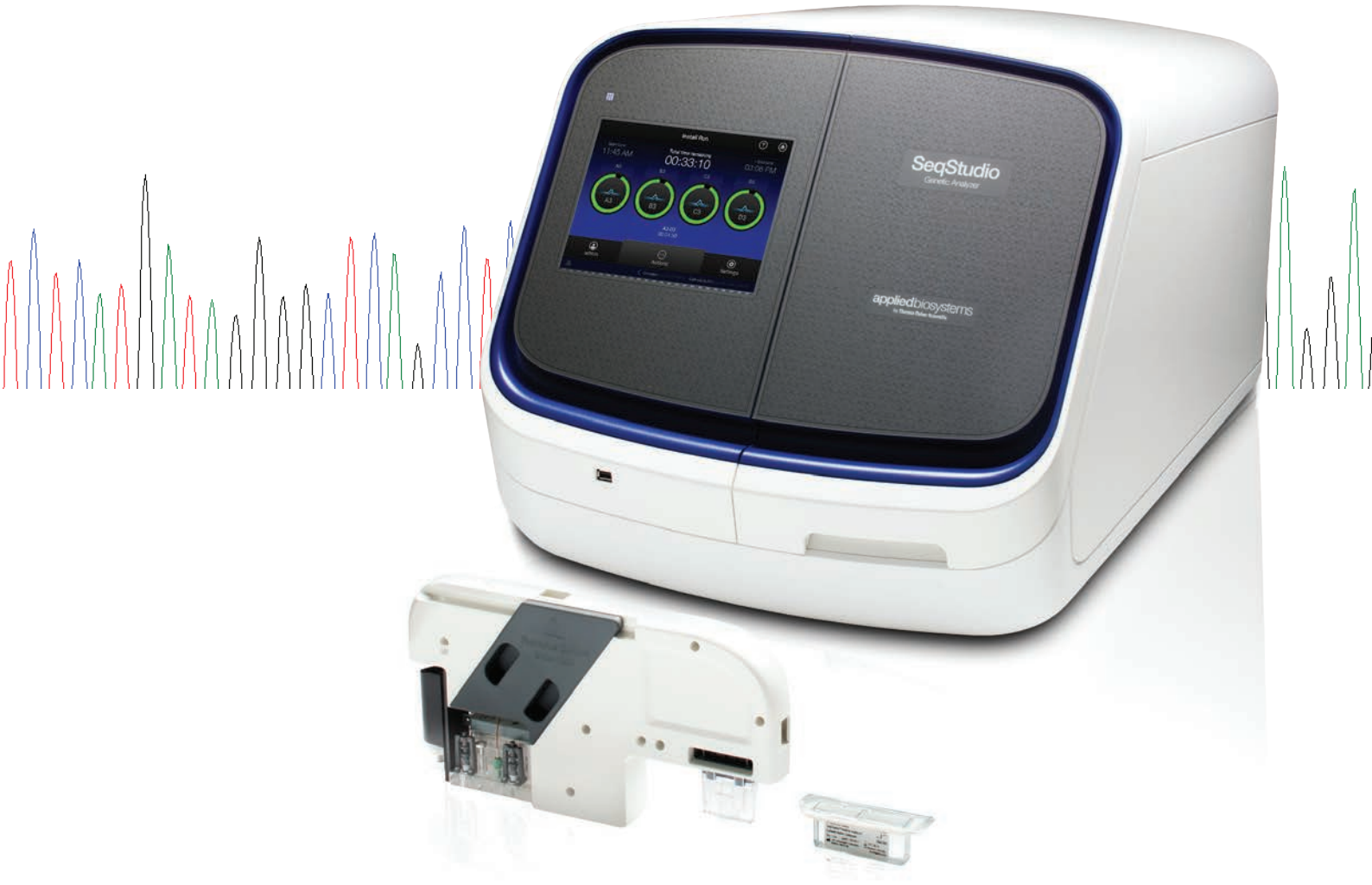




## Slides, Sections and Cells

The beautiful, intriguing and informative art of pathology

applied biosystems

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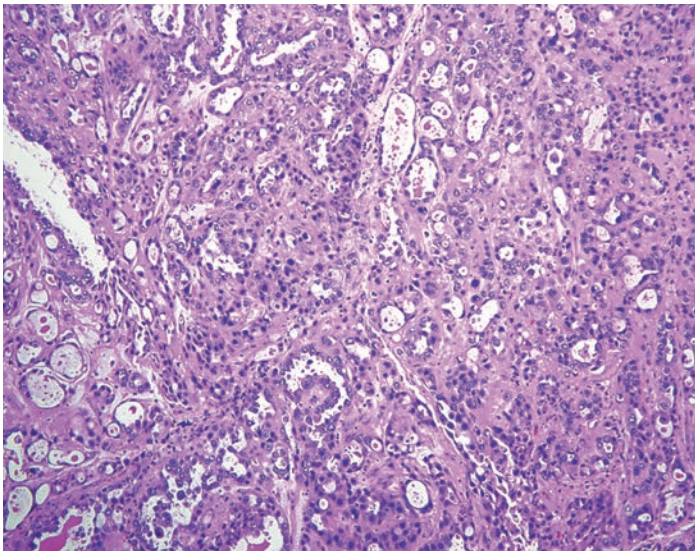
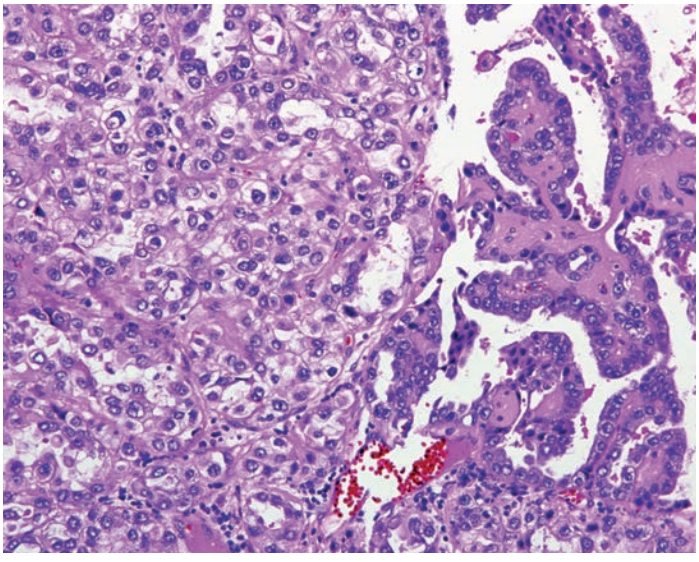
# Case of the Month





## *Ovarian Mass*

The hematoxylin-eosin-stained slides shown here were prepared from a 20 cm mass, partially cystic and partially solid, that was removed from the right ovary of a 59-year-old female. Immunohistochemically, the tumor was positive for napsin-A and PAX8, but negative for p53 and WT1.

*What is the most likely diagnosis?*

*a* High-grade serous carcinoma

*b* Clear cell carcinoma

*c* Mucinous cystadenocarcinoma

*d* Yolk sac tumor

## Answer to last issue's Case of the Month…

*C. Kaposi sarcoma.*

This tumor is composed of nodules of monomorphic spindle cells arranged in fascicles. Tumor cells form mitotic division figures and include rare entrapped inflammatory cells and erythrocytes. The expression of HHV-8 excludes other vascular neoplasms from the differential. This is the classic variant of Kaposi sarcoma, which has a predilection for the skin on the legs of elderly men. Angiosarcoma favors the face and scalp and is composed of widely infiltrative, hyperchromatic endothelial cells that form crack-like vascular spaces and are negative for HHV-8.

*Submitted by Garth Fraga, The University of Kansas, Kansas City, USA.*

**To register your guess for this month's case, please go to http://tp.txp.to/0617/case-of-the-month**
**We will reveal the answer in next month's issue!**

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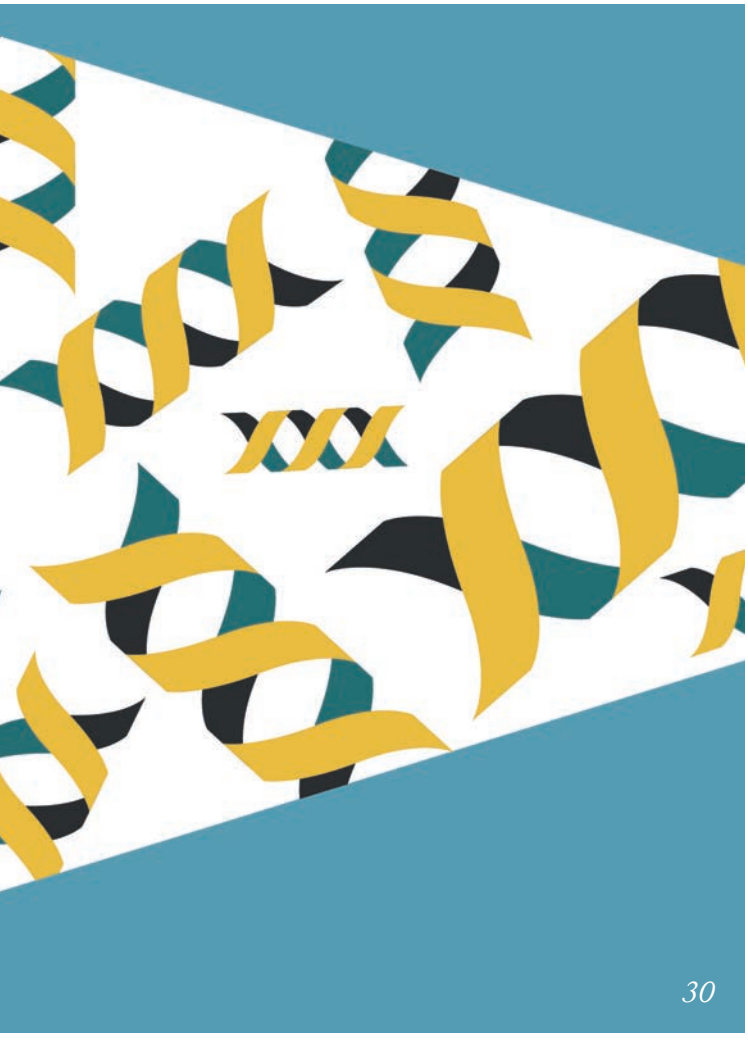




## On The Cover


*A whole slide image of a parasagittal section of a human fetus.*

## Upfront



## In My View


## Feature



## In Practice



## NextGen



## Profession



## Sitting Down With



the **Pathologist**

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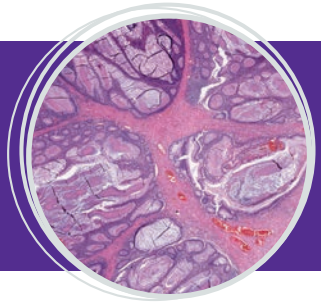

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# Not with a Whimper

*If you're worried about the future of pathology, read on...*



In June, I had the pleasure of visiting the oncology research and treatment mecca that is the Memorial Sloan Kettering Cancer Center, where I met with Michael Roehrl, Director of the Precision Pathology Center – and one of our Power List members. Our discussion took many twists and turns, but we kept returning to one topic: the future. In particular, the need for pathology to look forward and remain innovative and disruptive. We spoke at length about the lack of pathology subspecialization in Europe that is hindering progress and, worse still, good patient care; the inhibitive fear of diagnostic error; the urgent need for raised public awareness of pathology; the worrying acceptance of budgetary and resource constraints; resistance to innovation (look out for a follow-up article that I've already titled "Pathological Complacency"); and other specialties' critical dependence on pathologists and laboratory physicians, whom Roehrl aptly calls the "Physicians' Physician" (another article in the making...).

What struck me most during our conversation was Roehrl's passion for his profession, and the need for pathology to be a leader in innovation in the era of precision health care, especially when it comes to new technologies and disruptive theranostics. A challenge for pathology, he believes, has been predominantly caused by the reduced finances allocated to it, the falling numbers of trainees attracted to it, and sometimes a traditional mindset of those working within it. In short, we agreed that we need more people fighting for pathology.

Though my colleagues and I have been fortunate to work with many trailblazers and advocates, there aren't enough people who feel sufficiently empowered to talk about the value of pathology to medical colleagues, the budget controllers, government, the public and, dare I say it, to patients. Just to emphasize the point, I was talking with someone in industry recently who spoke of a customer – a very senior pathologist – who was normally openly communicative and articulate. Apparently, he became less talkative as he ascended a building in an elevator towards the floor containing the offices of C-suite executives where, on arrival, he "completely lost his voice." We must acknowledge that such failures in visibility come with consequences. I'll refer you to one of many articles that we have published on the subject (1). If you have any thoughts on this subject or if you would like to share your opinion, we would love to hear from you (edit@thepathologist.com).

I'll leave you with a quote from a survey of medical students that was presented at the 2017 annual congress of the Canadian Association of Pathologists: "Maybe you should take pathology out of the lecture titles so students aren't put off." We all need to work together to change this perception.

**Fedra Pavlou**
*Editor*

*Reference*

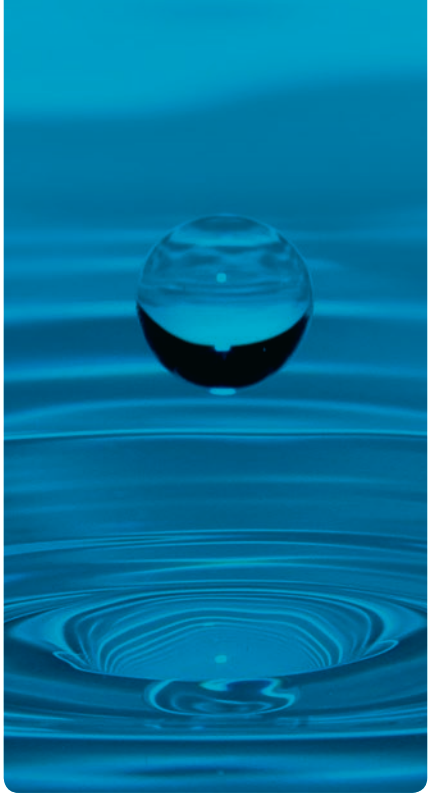
1. *JI López, "The Invisible Doctor", The Pathologist, 09, 46–48 (2015).*

# Upfront

*Reporting on research, innovations, policies and personalities that are shaping pathology today.*

*Do you want to share some interesting research or an issue that will impact pathology?*

*Email: edit@thepathologist.com*



## Small Samples; Big Promises

### Ultrasensitive mutation analysis could boost liquid biopsy

It's hard to look at a laboratory medicine journal without seeing the words "liquid biopsy" these days. Small wonder the technique is such a hit – it's simple, noninvasive, and makes use of emerging molecular techniques to tell us more than ever about the diseases our patients face. But with all of these advantages, liquid biopsy does face one challenge – sensitivity.

"The main issue with analyzing circulating cell-free DNA is that its concentration is low, and DNA of tumor origin is present at very low frequencies – sometimes only individual molecules," says Anders Ståhlberg, docent in molecular medicine at the University of Gothenburg's Sahlgrenska Cancer Center. "Standard techniques are not sensitive enough to find these rare molecules," he continues, "but with new approaches such as our SiMSen-Seq technique, this is now possible."

SiMSen-Seq allows the detection of circulating tumor DNA (ctDNA) in the blood with up to 1,000-fold more sensitivity than the methods currently in use. Ståhlberg and his colleagues accomplish this feat by adding a molecular barcoding step. "In molecular DNA barcoding, a unique sequence is added to each individual DNA molecule that enables us to track all sequencing reads back to the original DNA molecule. By aligning reads with the same barcode, it is then possible to differentiate between true mutations and those resulting from polymerase errors." SiMSEn-Seq is not the only liquid biopsy method to use barcoding, but Ståhlberg says that each method carries its own limitations. "Our contribution is that we managed to develop a cost-effective method that is simple to use, flexible to adjust, and can be used with minimal DNA input."

What are the researchers doing with the technique now? Ståhlberg outlines a number of clinical investigations applying ultrasensitive mutation detection to liquid biopsy, including patients with childhood sarcomas, melanomas and breast cancers. He and his team are also applying their approach to areas beyond cancer, including chronic obstructive pulmonary disease and immunological responses. Nonetheless, he warns against jumping into liquid biopsy too fast. "The potential of circulating cell-free DNA is very high, but validation studies are important to prove its clinical value. You may find mutations without a disease – so we need to learn how and when to perform this type of analysis."

Ståhlberg next plans to learn exactly which liquid biopsy applications gain the greatest clinical value from ultrasensitive mutation analysis. He and his colleagues have recently received funding from several collaborating organizations to start a translational genomics platform (3) working with liquid biopsies and ultrasensitive mutation analysis. And he's optimistic about the future of liquid biopsy: "By analyzing patient-specific mutations in blood plasma, we anticipate improvements in diagnosis, treatment selection, prognosis, treatment monitoring and relapse detection." *MS*

*References*

1. *A Ståhlberg et al., "Simple multiplexed PCR-based barcoding of DNA for ultrasensitive mutation detection by next-generation sequencing", Nat Protoc, 12, 664–682 (2017). PMID: 28253235.*
2. *A Ståhlberg et al., "Simple, multiplexed, PCR-based barcoding of DNA enables sensitive mutation detection in liquid biopsies using sequencing", Nucleic Acids Res, 44, e105 (2016). PMID: 27060140.*
3. *"Translational Genomics Platform" (2017). Available at: http://bit.ly/2rMqmwi. Accessed June 20, 2017.*

# Minimalist Monitoring

## A new antibody-based biosensor could facilitate drug monitoring in resource-poor areas

We are all well aware that patients in developing countries urgently need access to medications for chronic illnesses. But a discussion we have far less frequently is what happens once those patients receive the treatments they need.

"Monitoring drug concentration in patient blood is an important aspect of medical treatment to improve the efficiency of the drugs and decrease the side effects," says Lin Xue, a postdoctoral scholar at École Polytechnique Fédérale de Lausanne. But it's easier said than done; most monitoring calls for expensive equipment and complex facilities that may not be available in resource-poor areas. Often, patients have to stay close to the lab or hospital, infringing on their quality of life – and that's if they're able to access monitoring at all. Xue and his colleagues recognized the need for affordable point-of-care detection of drug concentrations in blood and developed a biosensor molecule made up of three components (1):

1. An antibody fragment that can bind the drug to be monitored,
2. The light-producing enzyme luciferase, and
3. A "tagging" molecule called SNAP-tag, which carries a fluorescent ligand that the antibody binds only when no drug is present.

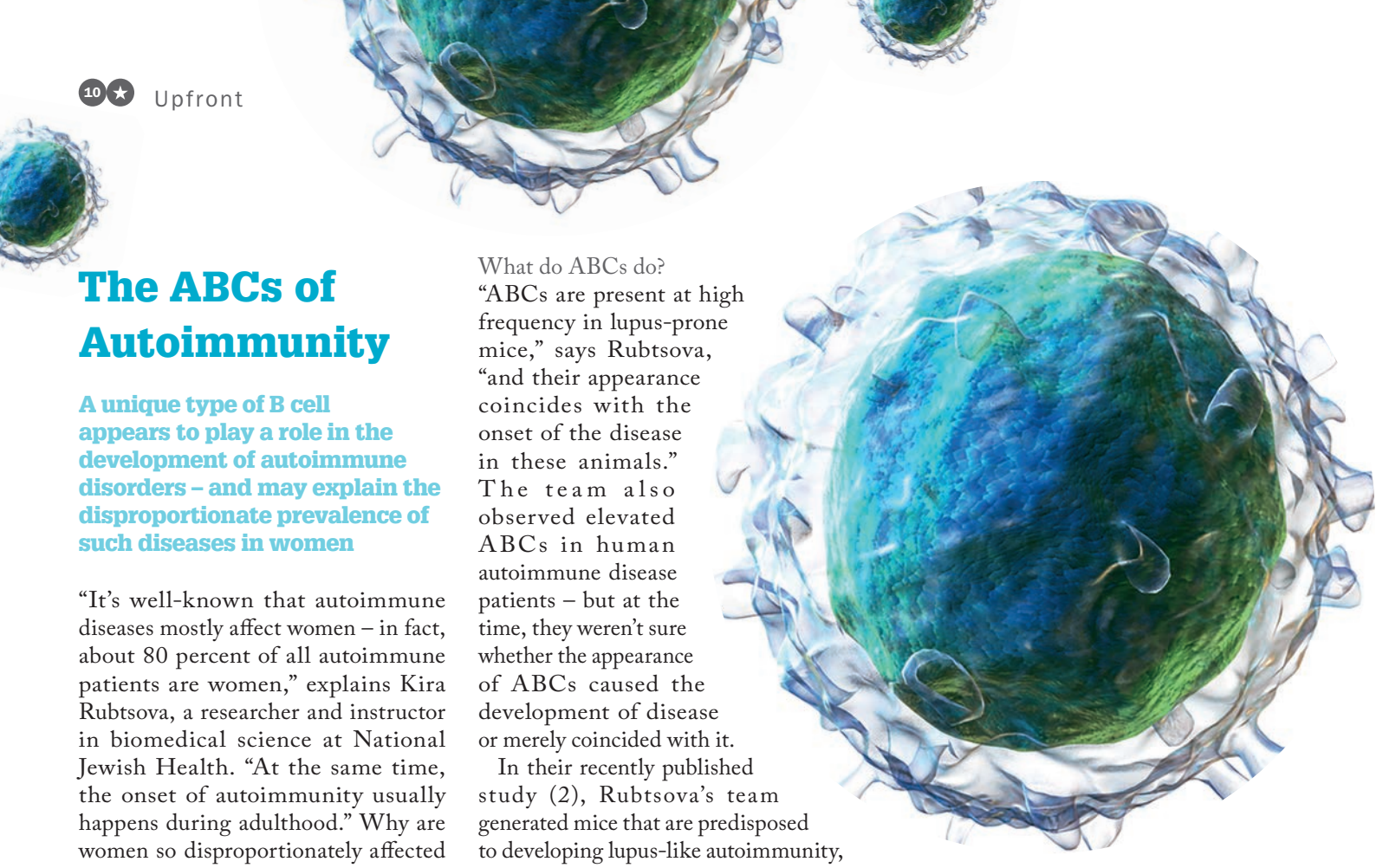
In the absence of the drug, the antibody and SNAP-tag bind, causing a reaction called "bioluminescent resonance energy transfer" that produces a red light. But as drug concentrations increase, the antibody preferentially binds to the drug, displacing the fluorescent ligand and causing the emission of a blue light instead. The result? A simple, measurable visual that indicates the amount of drug present in a patient's blood.

"The biosensors can be incorporated into paper-based point-of-care devices, which are cheap, portable, time-saving and easy to use. They could even be used by the non-experts, such as the patients themselves," says Xue and emphasizes that the system works with any antibody: "We can theoretically design antibody-based sensors towards an unlimited number of synthetic drugs." And the sensor's performance was independent of the antibody used – meaning that there's no costly, time-consuming optimization process needed to create a sensor for a new type of drug.

What would such a sensor look like in the clinic? Ideally, as simple as modern blood glucose meters, allowing patients to take a fingerprick sample and use a handheld reader, perhaps in conjunction with a smartphone. In fact, Xue's colleagues have already developed working prototypes of test strips and a reader, so the researchers are optimistic that their device should be available in the next few years. Ultimately, they'd also like to expand it; after all, says Xue, the need for monitoring doesn't stop at drug levels – he anticipates tests for pathogens, hormones, vitamins, and even biomarkers. *MS*

*Reference*

1. *L Xue et al., "Bioluminescent antibodies for point-of-care diagnostics", Angew Chem Int Ed Engl, 56, 7112–7116 (2017). PMID: 28510347.*



# The ABCs of Autoimmunity

**A unique type of B cell appears to play a role in the development of autoimmune disorders – and may explain the disproportionate prevalence of such diseases in women**

"It's well-known that autoimmune diseases mostly affect women – in fact, about 80 percent of all autoimmune patients are women," explains Kira Rubtsova, a researcher and instructor in biomedical science at National Jewish Health. "At the same time, the onset of autoimmunity usually happens during adulthood." Why are women so disproportionately affected by autoimmune issues? Rubtsova and her colleagues suspected that the female immune system undergoes changes with age that lead to the progression of autoimmunity – changes that the male immune system does not experience. But precisely what are those changes? In the quest to answer that question, Rubtsova's research group discovered age-associated B cells (ABCs).

### What are ABCs?

"ABCs are a unique subset of B cells that can be distinguished from other types by the expression of certain molecules. In particular, ABCs express the integrins CD11c and CD11b on their surfaces and contain high levels of transcription factor T-bet, none of which is expressed by other types of B cells." And when the researchers compared the gene expression profiles of ABCs with those of other B cells, they found hundreds of differentially expressed genes – indicating that ABCs have a unique phenotype and likely a unique function (1).

### What do ABCs do?

"ABCs are present at high frequency in lupus-prone mice," says Rubtsova, "and their appearance coincides with the onset of the disease in these animals." The team also observed elevated ABCs in human autoimmune disease patients – but at the time, they weren't sure whether the appearance of ABCs caused the development of disease or merely coincided with it.

In their recently published study (2), Rubtsova's team generated mice that are predisposed to developing lupus-like autoimmunity, but lack T-bet expression in B cells (meaning that they cannot generate ABCs). "We have followed the health conditions of these mice over time, comparing them with lupus-prone mice that can develop ABCs," she says. In the absence of ABCs, the lupus-prone mice were significantly less likely to develop disease. "These data led us to the conclusion that ABCs drive the onset of autoimmunity."

### Could ABCs be diagnostically useful?

"The presence of these cells can be used for diagnostic purposes," Rubtsova says. It's possible that, one day, patients suspected of autoimmune disorders could be tested for the presence of ABCs. And if the cells participate in the disease process, it's also possible that people at risk of developing such disorders might even be screened using ABCs one day, allowing doctors to spot autoimmune diseases before symptoms arise.

But Rubtsova also wants to highlight the therapeutic potential: "These cells can be used as targets for the development of novel therapies that could cure autoimmunity. Because our results indicate that ABCs represent the pathogenic subset of cells, their removal may lead to the amelioration of disease." One of her team's next goals is to develop a drug capable of depleting or inactivating ABCs.

In the meantime, many questions remain. How do ABCs promote the development of autoimmunity? Why is T-bet expression so critical for the development of these cells? And why are females more predisposed to developing ABCs and autoimmune diseases? *MS*

*References*

1. *K Rubtsova et al., "Age-associated B cells: a T-bet–dependent effector with roles in protective and pathogenic immunity", J Immunol, 195, 1933–1937 (2015). PMID: 26297793.*
2. *K Rubtsova et al., "B cells expressing the transcription factor T-bet drive lupus-like autoimmunity", J Clin Invest, 127, 1392–1404 (2017). PMID: 28240602.*

# Parkinson's Predictions

**Could new biomarkers predict which Parkinson's disease patients are most likely to suffer early or severe cognitive decline?**

"Significant cognitive impairment is a dreaded outcome for Parkinson's disease patients," says Daniel Weintraub, Professor of Psychiatry at the University of Pennsylvania's Perelman School of Medicine. "We need to do all that we can to understand this key non-motor feature of the disease and to develop better treatments and preventive strategies." To that end, Weintraub and his colleagues are investigating the neurobiology of cognitive changes – and the effort has led to a new set of biomarkers that could help predict which patients are most likely to suffer cognitive decline (1).

The team found four distinct biomarkers, each independently associated with cognitive impairment over the study's three-year duration: dopamine deficiency, lower levels of β-amyloid protein in the cerebrospinal fluid (CSF), and single nucleotide polymorphisms in the *COMT* and *BDNF* genes. In addition, decreased brain volume or thickness upon imaging was also associated with impairment. "We now have evidence that changes in the dopamine system, Alzheimer's disease pathology, and unrelated genetic factors all contribute independently to cognitive decline in Parkinson's disease."

So is there potential for a clinical test – for diagnosis, prognosis, or therapeutic monitoring? Potentially all of the above, says Weintraub. "It is relatively simple and inexpensive to obtain blood (for genotyping), a brain structural MRI (for atrophy and thickness) and CSF (for Alzheimer's disease biomarkers). Any of these biomarkers, singly or as a group, could help predict present and future cognitive decline." As to monitoring treatments, the potential is certainly there, but Weintraub notes that additional studies will need to determine whether the biomarkers predict response to, or are affected by, cognitive-enhancing medications.

If such a test were developed, it would be of greatest use in patients with early-stage Parkinson's disease, before significant cognitive impairment has occurred. Weintraub foresees an additional application, though. "It will also be helpful when we have pathology-specific treatments, such as amyloid- or synuclein-focused compounds, when we need to know the specific neural substrate for cognitive impairment at an individual level." The first step along that path is to follow a larger number of patients for a longer period of time, so that the researchers can see whether or not these same factors predict not just cognitive decline over a three-year period, but also conversion to dementia – an even more serious outcome. *MS*


*Reference*

1. *C Caspell-Garcia et al., "Multiple modality biomarker prediction of cognitive impairment in prospectively followed de novo Parkinson disease", PLoS One, 12, e0175674 (2017). PMID: 28520803.*


| *Biomarker* | *Definition of cognitive impairment* | |
|---|---|---|
| | MoCA <26, or change in MoCA score | Site investigator diagnosis (PD-MCI or PDD) |
| DAT imaging | – | ↓ ipsilateral caudate*<br>↓ contralateral caudate§<br>↓ contralateral putamen§ |
| CSF | – | ↓ CSF Aβ 1-42* |
| Structural MRI (volume) | ↓ entorhinal*<br>↓ superior temporal*§<br>↓ caudal middle frontal§<br>↓ lateral orbitofrontal§<br>↓ superior parietal§ | ↓ lateral occipital*<br>↓ lateral orbitofrontal*<br>↓ fusiform*§<br>↓ superior temporal§ |
| Structural MRI (thickness) | ↓ precentral§ | ↑ caudal anterior cingulate§<br>↓ fusiform§ |
| DTI (FA) | – | – |
| DTI (MD) | – | ↓ inferior cerebellar peduncle* |
| Genetics | – | COMT V158M (V/V)<br>BDNF V66M (V/V) |

A summary of biomarkers and their use in detecting cognitive impairment.

# 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*

# The Importance of Chiral Metabolomics

**Chiral amino acids, metabolites long overlooked as "unnatural," are now under the spotlight – as biomarkers for kidney disease**

*By Tomonori Kimura, Department of Nephrology, Osaka University Graduate School of Medicine, Osaka, Japan*

Chronic kidney disease (CKD) is a global health problem. The number of patients with worsening kidney function who eventually need costly kidney replacement therapy or transplantation is increasing. In addition, the risk of life-threatening cardiovascular diseases increases with CKD progression. Preventing patients from progressing to end-stage kidney disease (ESKD) is therefore critical, but unfortunately there are no effective methods to predict progression. Currently, we rely on kidney functions estimated from serum creatinine and some additional information, such as proteinuria, but these are insufficient. Naturally, nephrologists are searching for better biomarkers.

Could amino acids, those vital components of human bodies, help provide the answer? Amino acid levels are influenced by the functions of many organs; kidneys, for instance, regulate the body's amino acid balances via reabsorption. Scientists have been studying amino acids for over a century – but because people only detected L-forms in nature, D-amino acids were regarded as unnatural and were not studied vigorously. Eventually, sporadic reports arose of the presence of D-amino acids, including in the blood of patients with kidney disease. Some studies also indicated physiological roles for D-amino acids, but once again these reports were sporadic – mainly because of the measurement challenge. Typically, D-amino acids are present in human bodies at trace levels, and the chemically similar nature of amino acid enantiomers makes it difficult to separate them and measure them simultaneously. And because reliable methods to measure D-amino acids are lacking, their functions and presence in tissues have remained a mystery.

It is only recently that methods have been devised to measure D-amino acids precisely via a metabolomic approach. Kenji Hamase and his colleagues went to great lengths to develop a metabolomic platform – based on micro-2D-HPLC – that can precisely detect whole sets of chiral amino acids from human samples (1). In the first dimension, labelled amino acids are separated by reverse-phase separation. Then, the fraction of each amino acid is automatically transferred to the enantioselective (chiral-selective) column for chiral separation. The 2D-HPLC system is powerful enough to quantitatively detect all amino acid enantiomers from clinical samples ranging from around 1 fmol to 100 pmol.

Our research group used chiral amino acid metabolomic profiling to search for prognostic biomarkers of CKD. Our study revealed that D-amino acids, particularly D-serine and D-asparagine, were robustly associated with the progression of CKD to ESKD (2). The risk of progression was elevated from two- to four-fold in those with higher levels – and this trend is seen only in D-forms. The fact that just a trace portion of amino acids have a stronger relationship with disease processes and prognosis strongly supports the importance of chiral separations.

A D-amino acid test could provide a powerful tool for clinicians, helping them identify high-risk CKD patients

for intensive care. The development of a device suitable for clinical use – designed to increase throughput – is currently underway. Another important direction for the future will be more detailed research to study the physiology and metabolism of D-amino acids – both of which are poorly understood – so that we can enrich our understanding of kidney diseases. Through chiral metabolomics, I believe that the mysterious world of D-amino acids will turn out to be a fruitful one for clinicians.

*References*

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2. *T Kimura et al., Sci Rep, 6, 26137 (2016). PMID: 27188851.*

# Embracing the Proteogenomic Toolkit

## To win the war on cancer, we need to put proteomics on an equal footing with genomics

*By Andreas Hühmer, Director, Thermo Fisher Scientific, USA*

Advances in our understanding of cancer biology through gene expression analysis have resulted in major steps towards the goals of reliable and effective cancer diagnosis, prognosis and treatment. But despite the progress we've made over the past few decades, many would justifiably argue that genomics has not fully lived up to its promise.

Although a number of cancer-driving gene mutations have been identified through the genomic characterization of tumor tissue by large-scale projects such as the Cancer Genome Atlas, the widespread identification of targetable cancer drivers remains a significant hurdle. For metastatic breast cancer, for instance, few validated oncogenic drivers exist (1). Moreover, establishing whether gene mutations are cancer "drivers" or "passengers" continues to be a challenge – and is difficult to determine based on genomic assessment alone.

Genomics has taught us that cancer is far more complex than we previously thought. The tumor microenvironment is highly heterogeneous, with significant variability even between individual cancer cells (2). This complexity is compounded by the fact that cancer is dynamic; taking a tumor sample and sequencing its genetic contents merely produces a snapshot, not the blueprints for future tumor growth. The apparent lack of correlation between the genome and phenome highlights the need for a complementary proteomic approach to unravel cancer's complexity.

Meanwhile, our ability to map out the proteomic landscape within tumor tissue has steadily grown over the past two decades. Advances in mass spectrometry and informatics now allow us to study protein samples on an unprecedented scale. The latest liquid chromatography-mass spectrometry (LC-MS) technologies, coupled with new multiplexed proteomics approaches based on isobaric labeling and advances in data processing, are leading to improvements in the depth and speed of quantitative proteome profiling – all while using ever smaller sample volumes (3).

But it's when these two approaches are used in combination that we can make the most progress. Proteomics techniques are now being used alongside genomic analysis to help unlock new cancer immunotherapies far more quickly than conventional approaches (4). Traditionally, the search for targetable cancer antigens was a time-consuming and error-prone process, involving DNA sequencing and mutation prediction algorithms, followed by large-scale immunological assays. Using mass spectrometry to profile peptides directly, we can reduce that timeline to a matter of weeks.

Advances in proteomics technologies are also driving improvements in cancer biomarker discovery. Recently, groundbreaking research by the Karolinska Institute in Sweden demonstrated the potential of integrating both protein and genetic markers in a single test for prostate cancer (5). And one goal of the US' National Cancer Moonshot program's Blood Profiling Atlas project is to develop a readily accessible database of blood biomarkers that will make it easier for oncologists to diagnose patients using liquid biopsies.

Genomics will continue to play an important role in cancer research. However, it is becoming clear that gene expression analysis alone is unable to sufficiently advance our understanding of cancer biology necessary for truly effective patient stratification and personalized therapy. A decade of technological development has made proteomics research-ready; we must now fully use the whole proteogenomics toolkit to truly make inroads on the fight against cancer.

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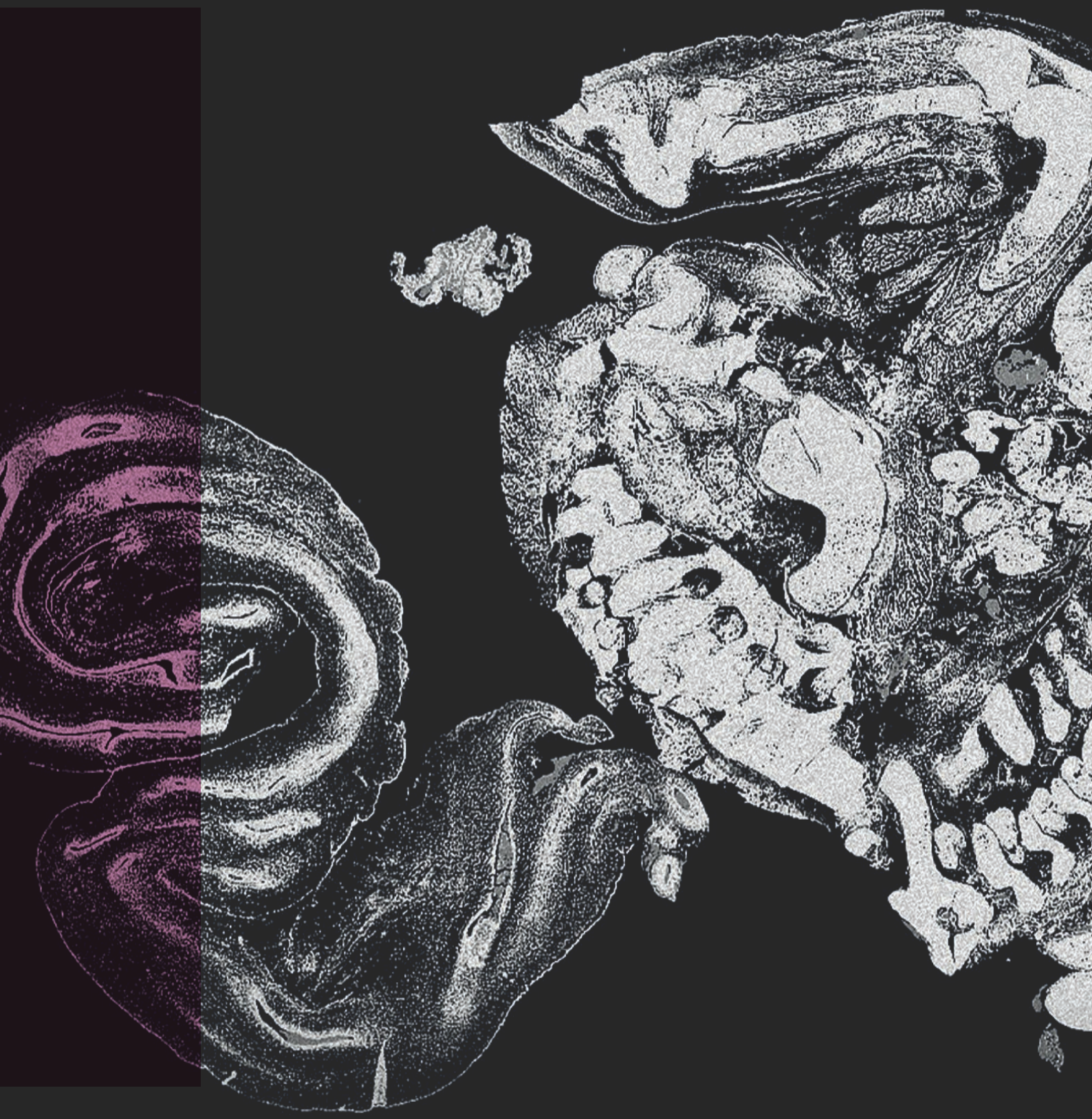
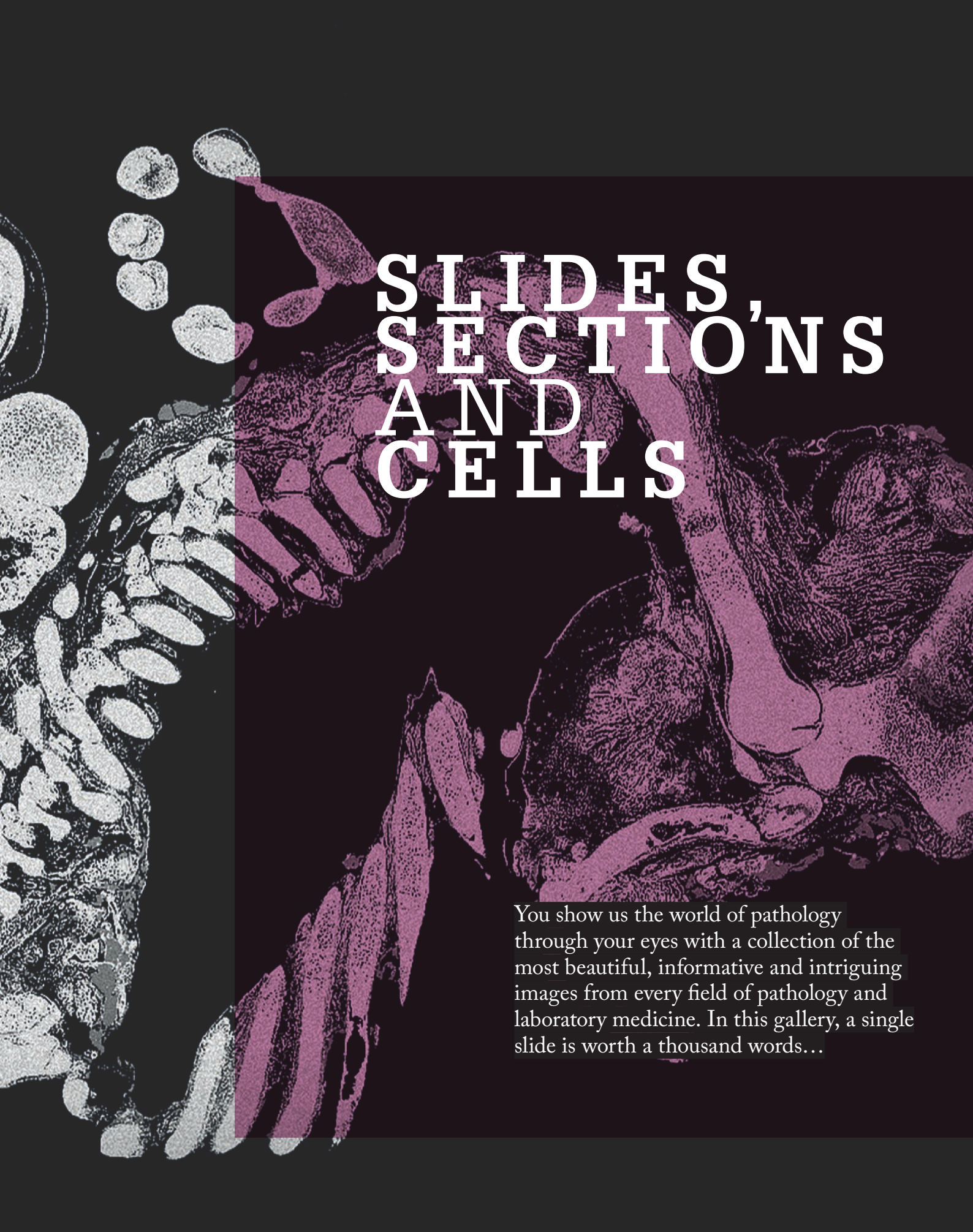
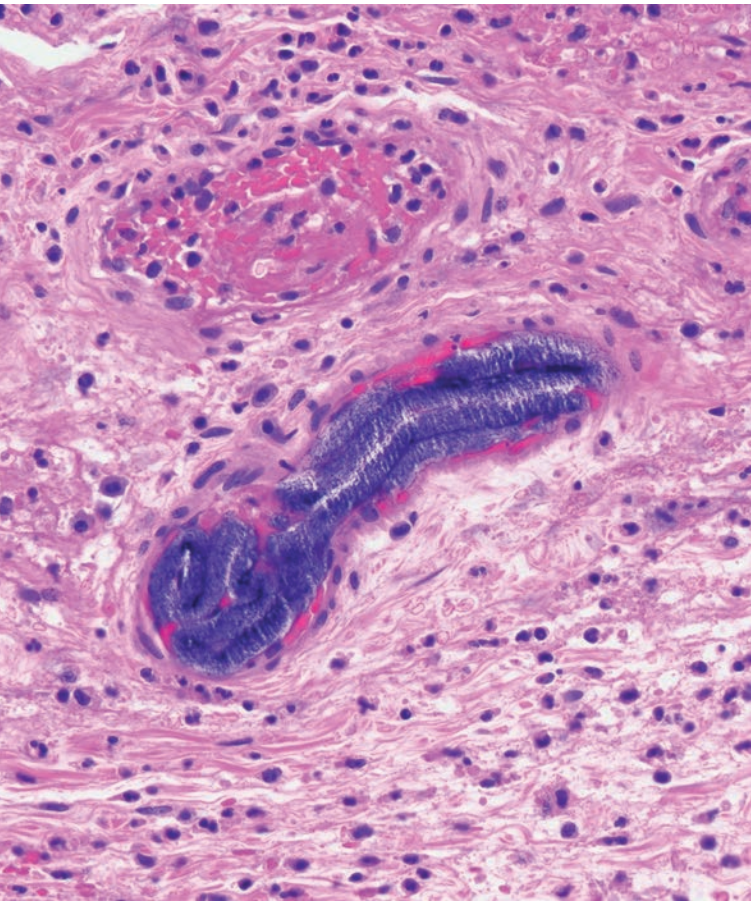
5. *H Schröder et al., Lancet, 384, 2027–2035 (2014). PMID: 25108889.*

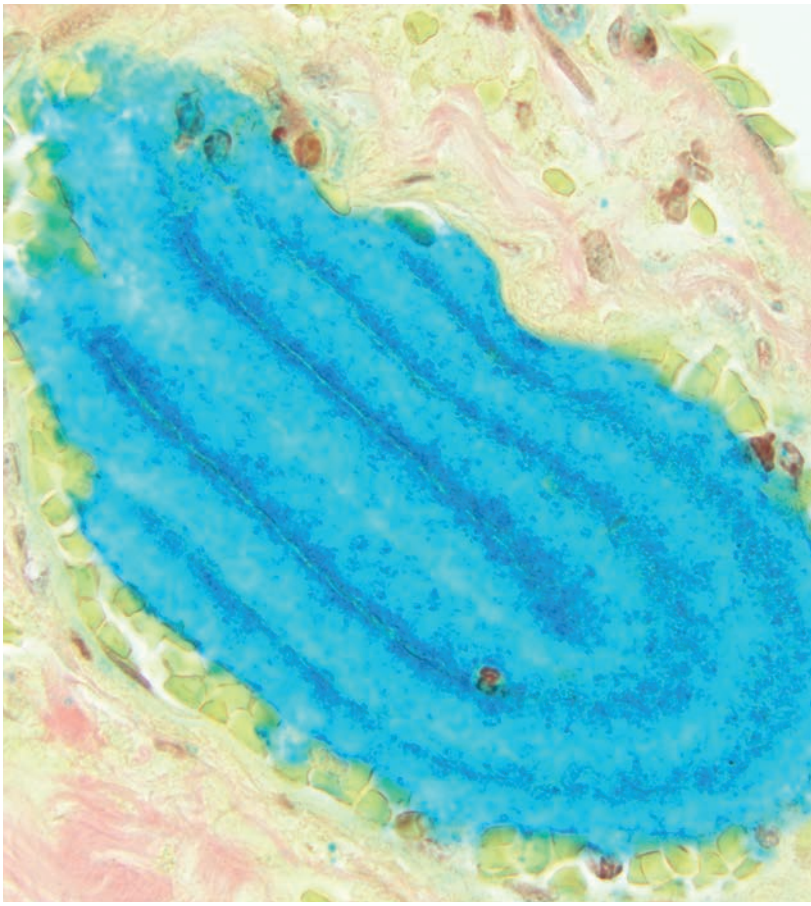




# SLIDES, SECTIONS AND CELLS

You show us the world of pathology through your eyes with a collection of the most beautiful, informative and intriguing images from every field of pathology and laboratory medicine. In this gallery, a single slide is worth a thousand words…





## HYDROPHILIC POLYMERS

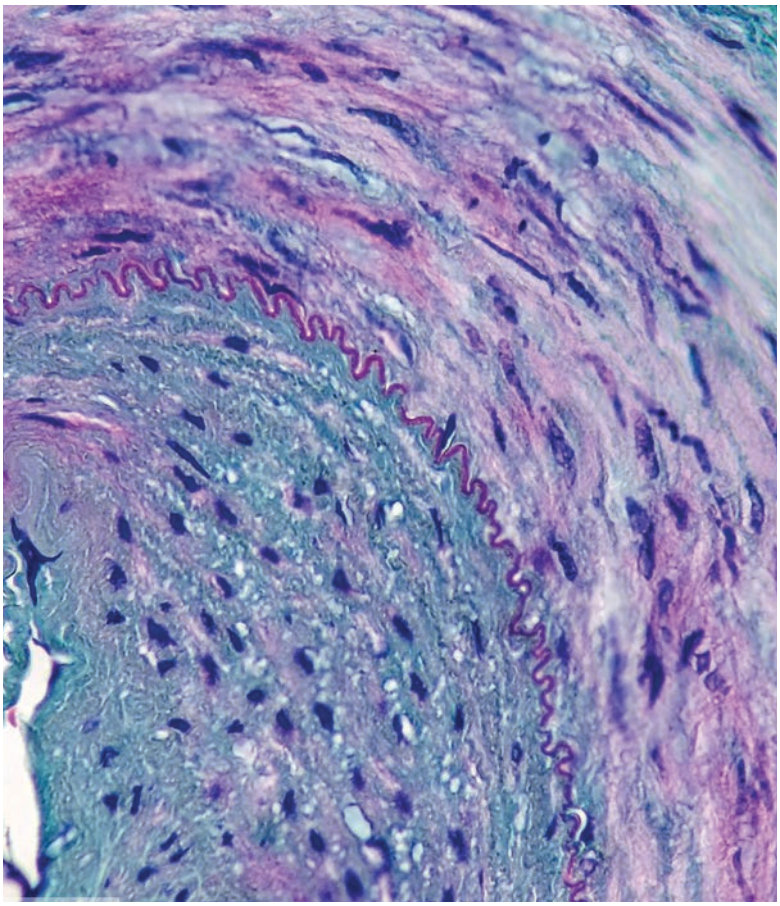
Hydrophilic polymers identified from a case of ischemic colitis in a patient with an aortic aneurysm repair. The polymers display a serpiginous configuration within vessels and a fuzzy and stippled basophilia on H&E (top left) and are turquoise on colloidal iron (top right).

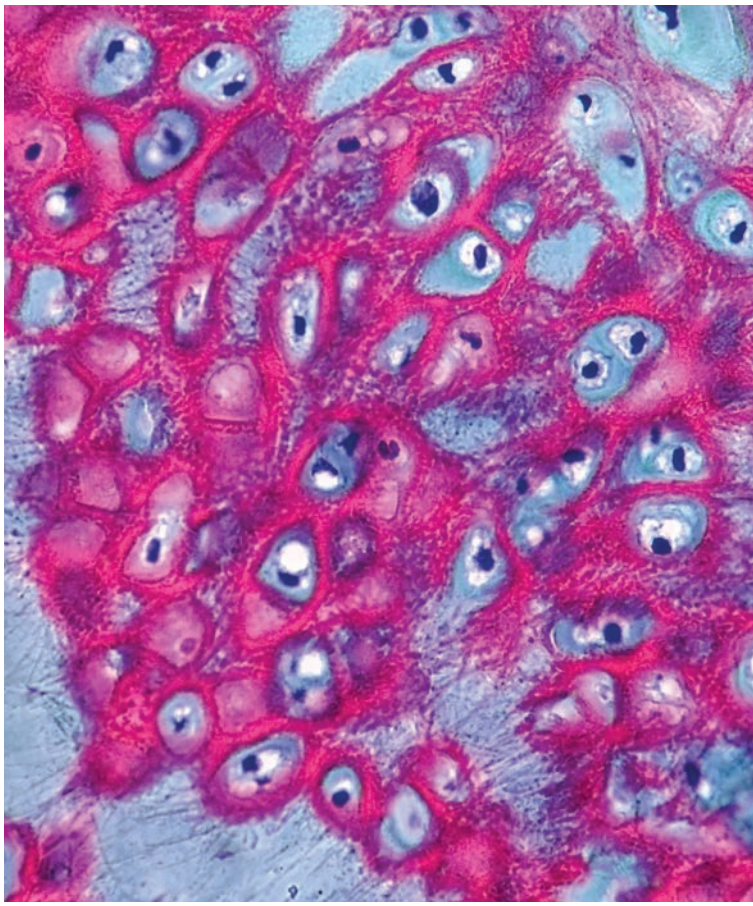
*Christina Arnold, The Ohio State University, USA.*

## BLOOMING CONDYLOMA

This piece was generated by Adobe Photoshop from a condyloma acuminatum, or genital wart (bottom).

*Maha Abdulla, The University of Kanasas School of Medicine, USA.*





## ELASTIC CARTILAGE

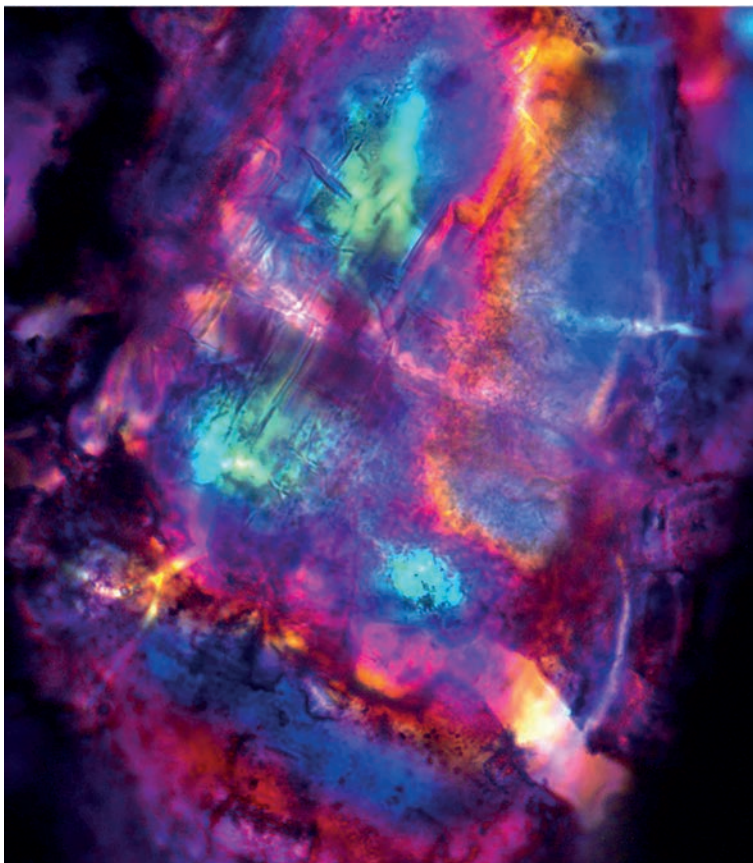
These images of elastic cartilage are stained by H&E and Nejib's stain (top).

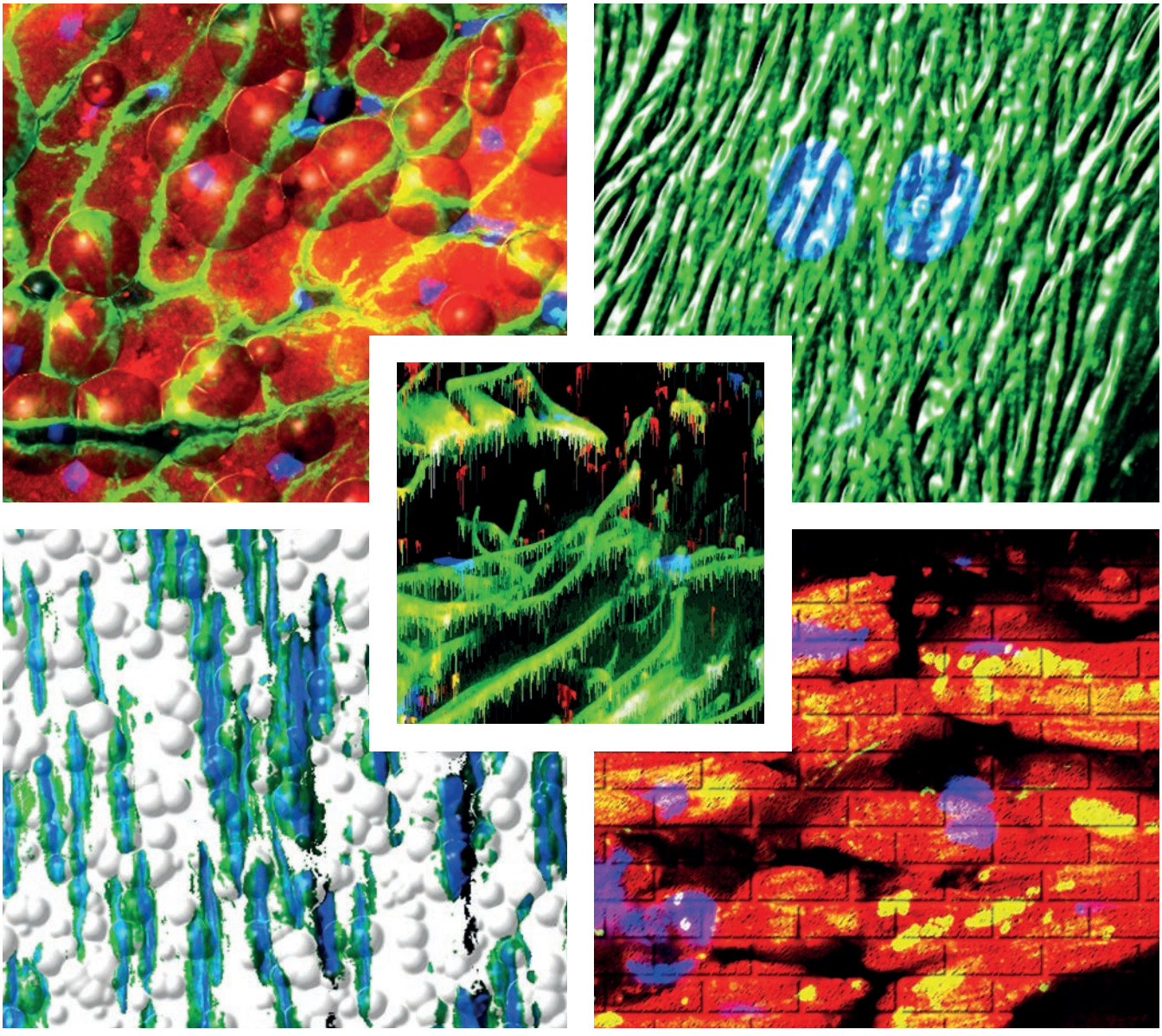
*Luis Humberto Cruz, Mother and Child Hospital, Irapuato, Mexico.*

## MICROCOSMS WITHIN A CRYSTALLURIA

This urinary cytology image was captured at 1000x by polarized light microscopy using an immersion objective and Papanicolaou stain (bottom).

*JD Prieto, UGC Provincial de Anatomía Patológica and LABCO Pathology, Málaga, Spain.*





## THE ART OF FLUORESCENCE DECONVOLUTION IMAGING

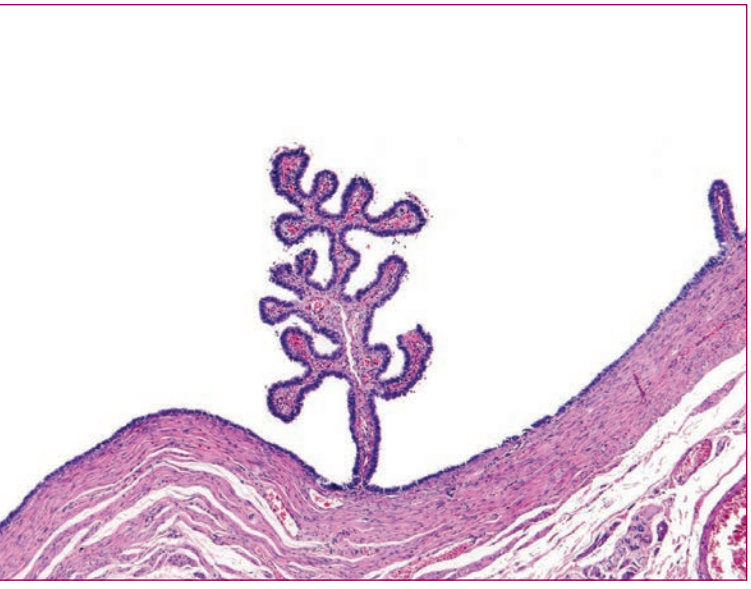
A series of artistic images created using fluorescence deconvolution microscopy. Clockwise from top left: "Bubbling" dystrophin in heart; "plastic" cardiomyocytes; a "wall" of myocardium; smooth muscle "bubbles"; and "dripping" connective tissue in skin.

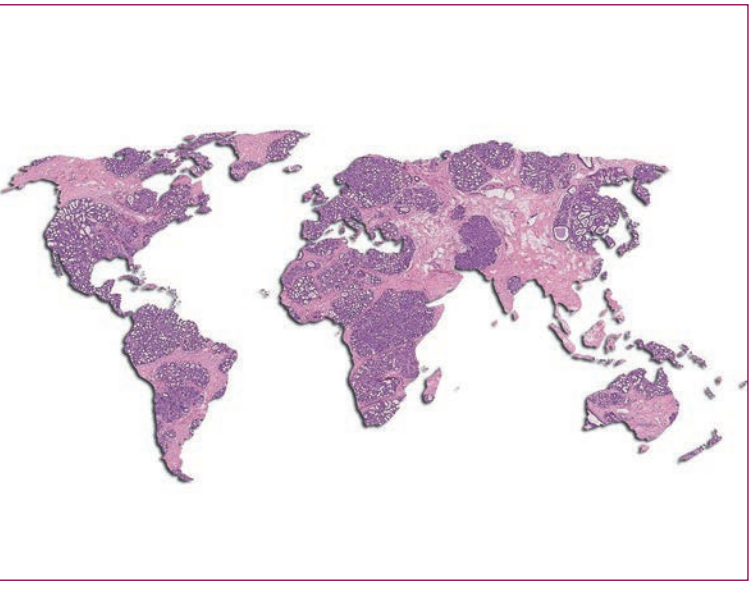
*Brian J. Poindexter and Roger J. Bick, Multi-User Fluorescence Imaging and Microscopy Core Lab, UT McGovern Medical School, USA.*

**PARASAGITTAL SECTION OF FETUS**

A whole slide image of a parasagittal section of the human fetus. Original magnification 0.45x.

*Vamsi Parini, Loyola University Medical Center, USA.*






## IN THE DESERT OF PARAFFIN ISLAND

A lonely papillary projection found amidst tubal epithelia in a dilated hydrosalpinx specimen (top left).

*Marcelo Balancin, Pathologist, São Paulo, Brazil.*

## THE WIDE WORLD OF HISTOPATHOLOGY

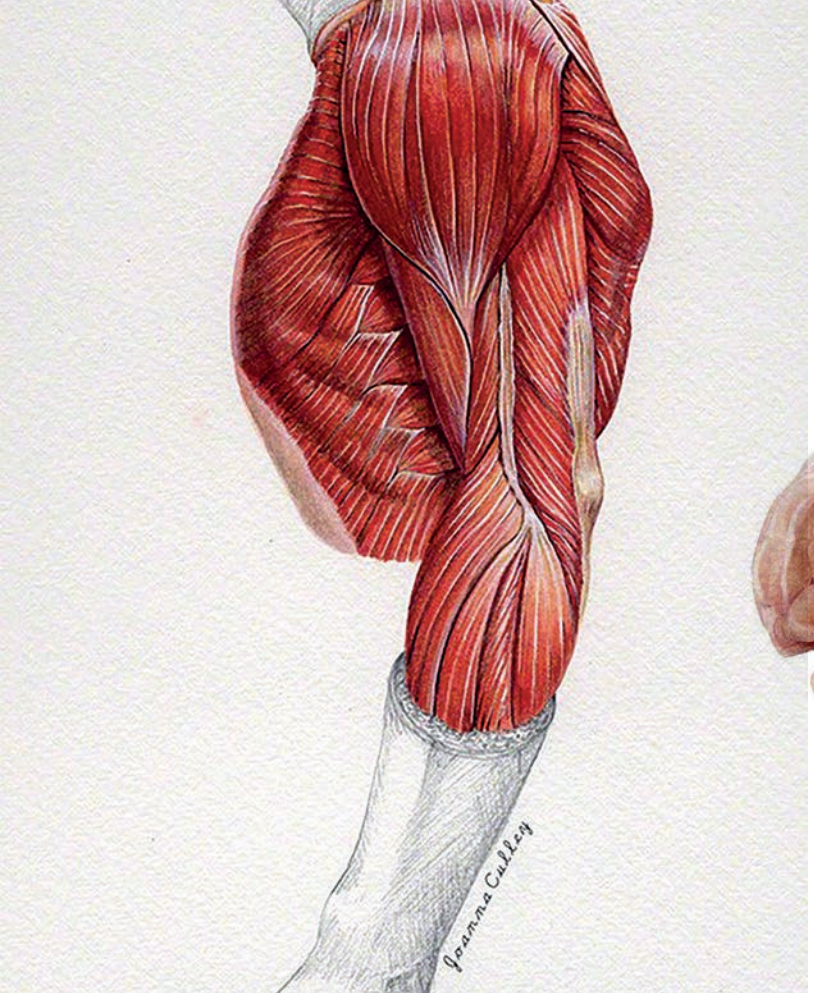
This world map (bottom left) offers a creative take on the vast scope of tissue pathology.

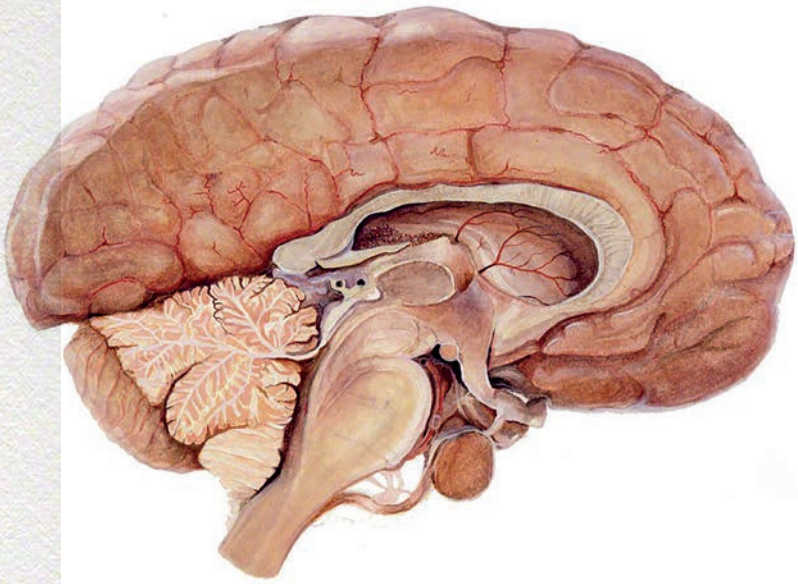
*Nathan Swailes, @ihearthisto*

## A MICROSCOPE IS AWESOME

This mixed-media piece (right) was created by the nine-year-old daughter of a pathologist – perhaps a future pathologist herself!

*Mishal Azam, Edgbaston High School for Girls, Birmingham, UK.*

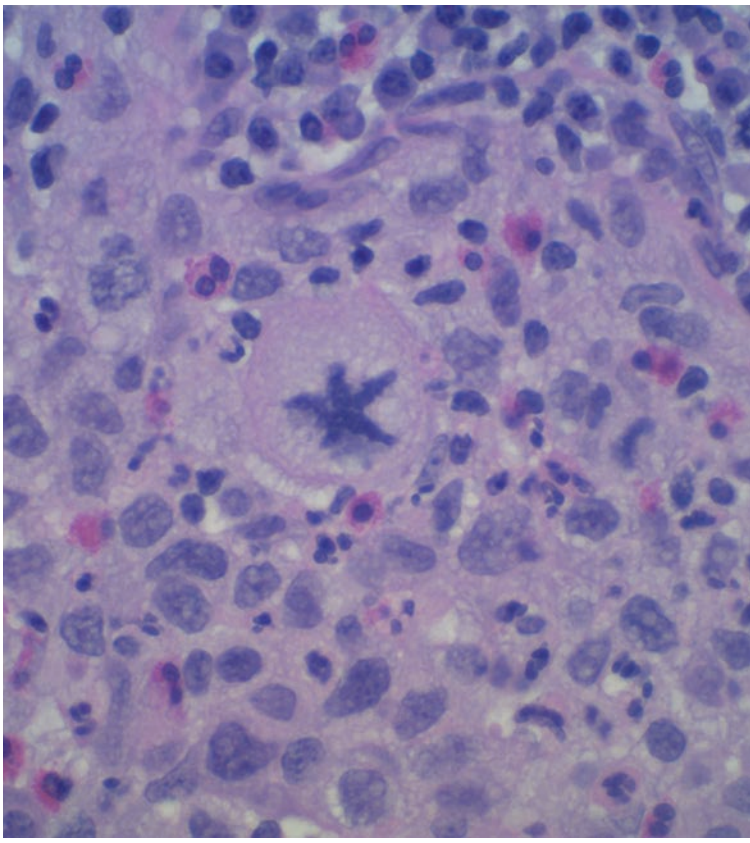


## WORKS IN WATERCOLOR

Musculature of the arm and chest (top left) and brain showing an enlarged pituitary gland (top right).

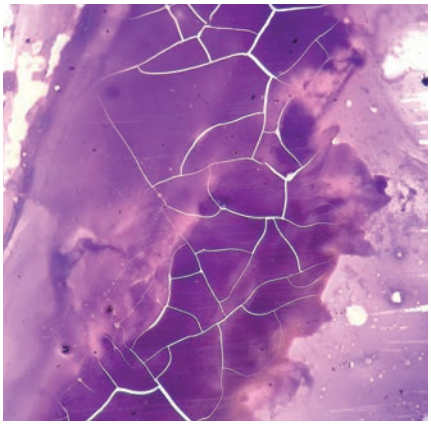
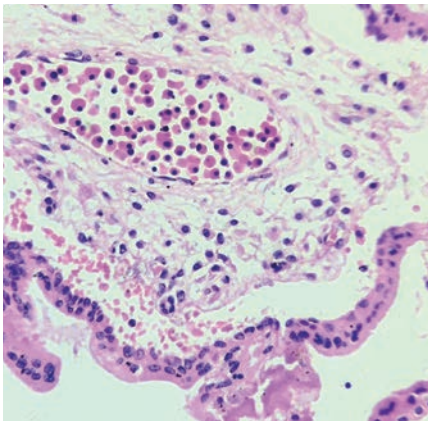
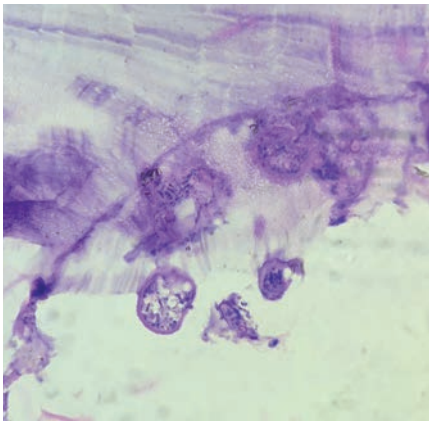
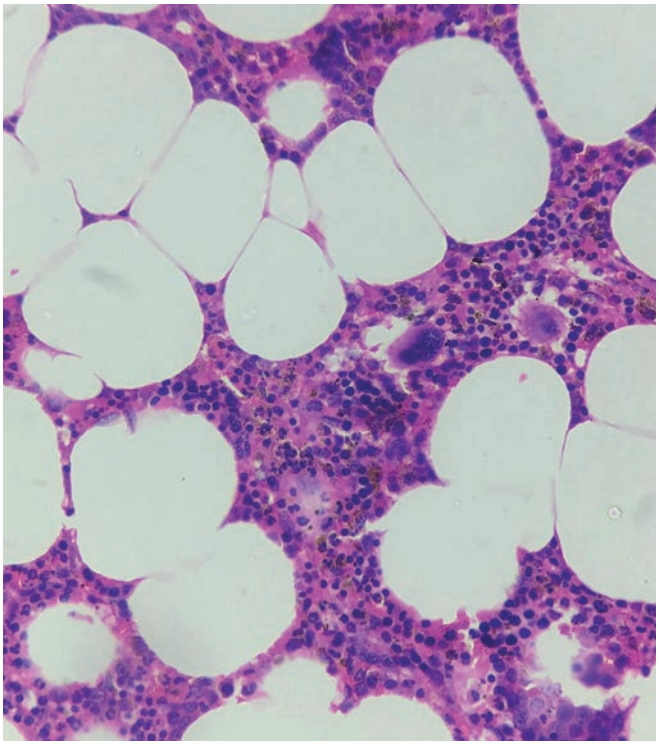
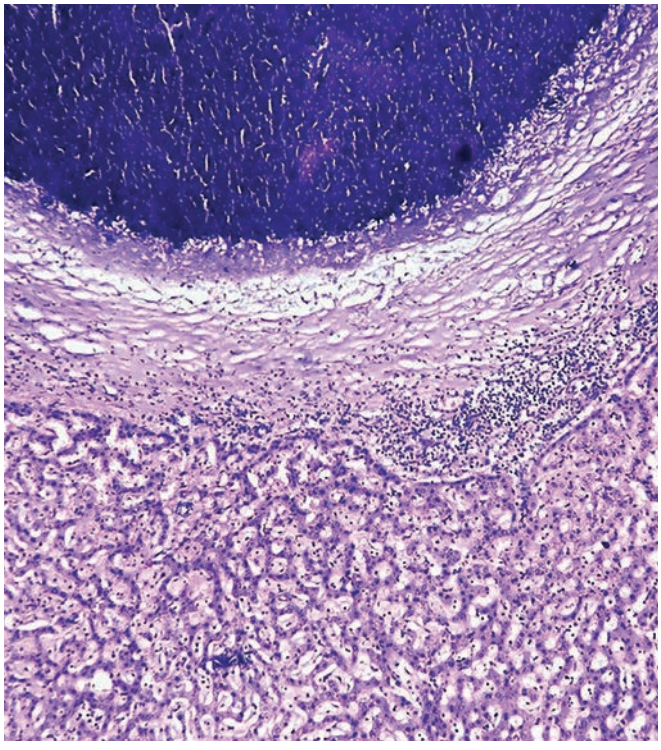
*Joanna Culley, Medical Artist, medical-artist.com*



## STARFISH

An atypical mitotic figure identified in high-grade invasive urothelial carcinoma (bottom).

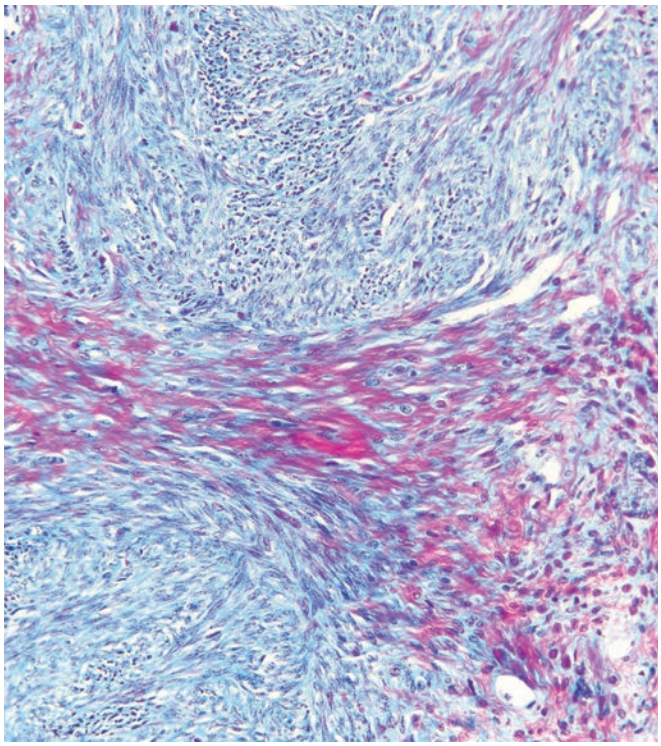
*Craig Hart, York Pathology Associates LLC, USA.*

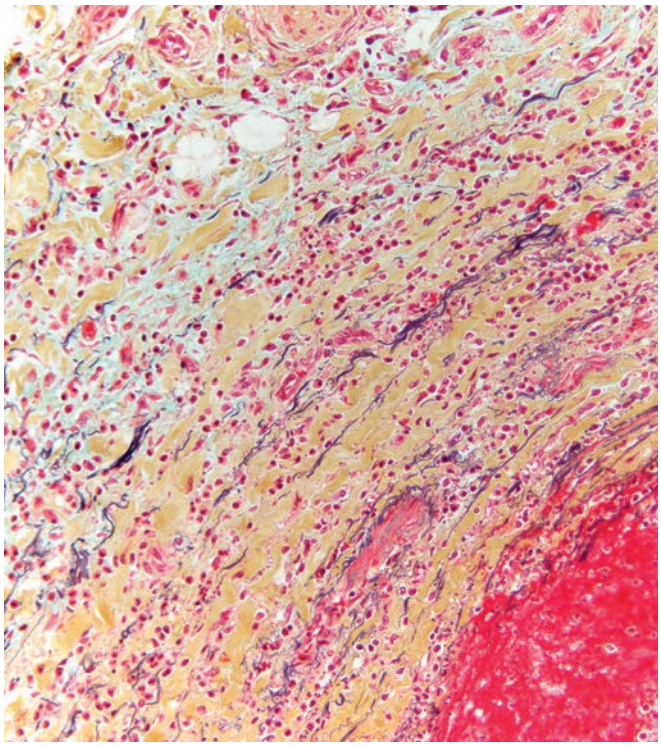






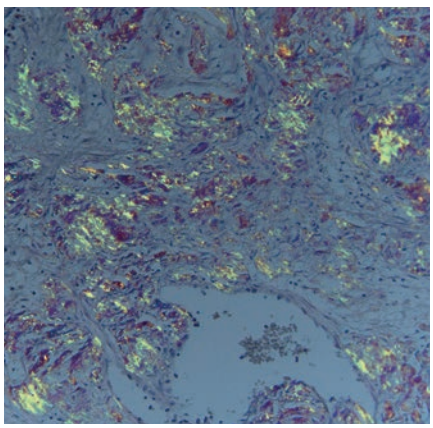
## THE BEAUTY OF PATHOLOGY

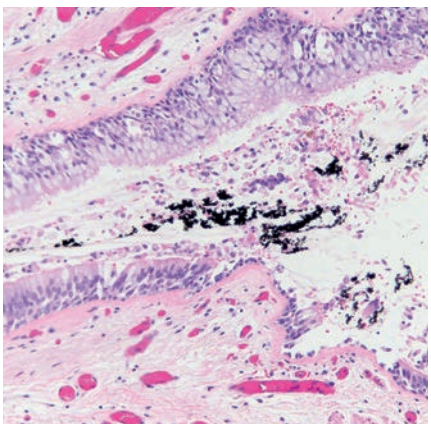
Clockwise from top left: The cracked-glass appearance of a colloid; nucleated red blood cells in a placental villus; a hydatid daughter cyst; a calcified liver cyst; and a myelolipoma.

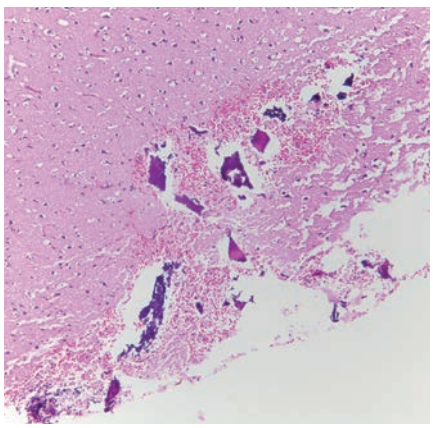
*Sunil Swami, India.*







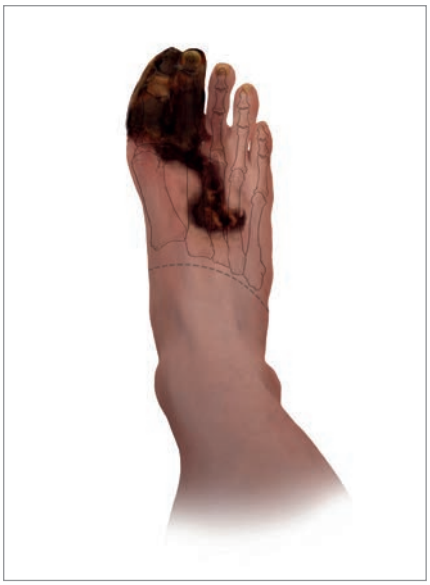


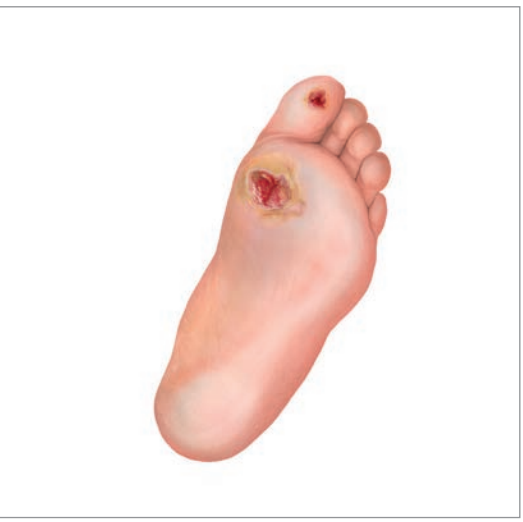


## DEATH UNDER GLASS

Clockwise from top left: Trichrome stain of muscle (blue: connective tissue, red: muscle fibers); pentachrome stain (black: nuclei and elastic fibers, yellow: collagen and reticular fibers, red: muscle, blue: mucin, bright red: fibrin); a shotgun blast to the head (pink: brain tissue, purple: bone); respiratory tract with soot; and amyloidosis, recognizable by its unique apple-green color.

*Marianne Hamel and Nikki Johnson, The Death Under Glass Project, USA.*

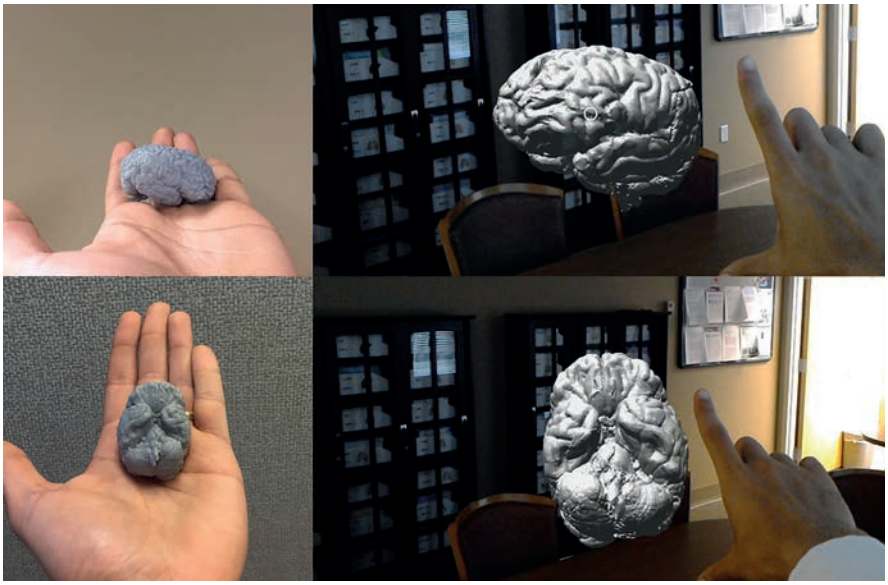




## PODIATRIC PATHOLOGY

Wet and dry gangrene of the foot (top left) and diabetic foot ulcers (bottom left).

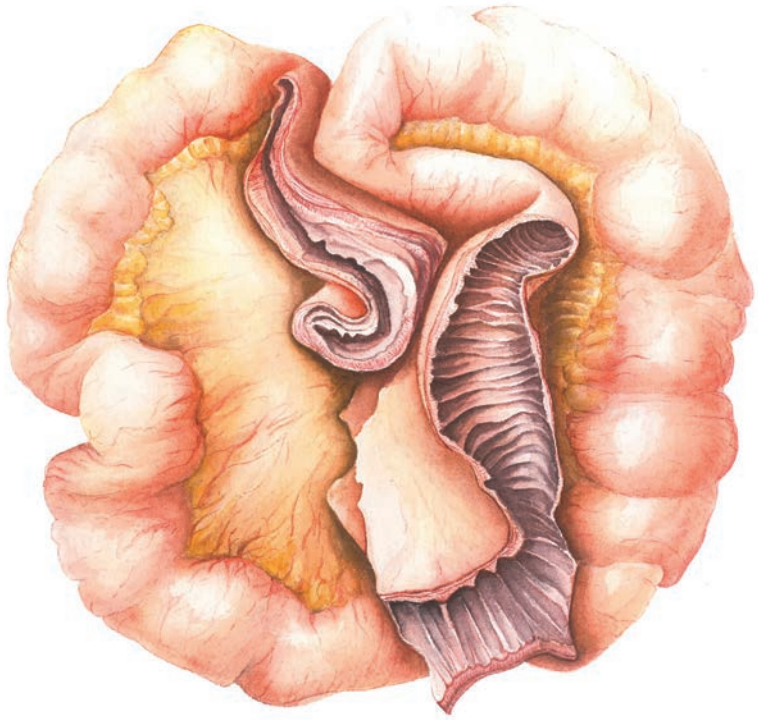
*Merlin Strangeway, Medical Artists' Association of Great Britain, merlinevans.com*



## HOW IMAGES ARE MADE

A composite of a human brain, both as a handheld 3D printout and displayed as a 3D hologram viewed in the Microsoft HoloLens (top right).

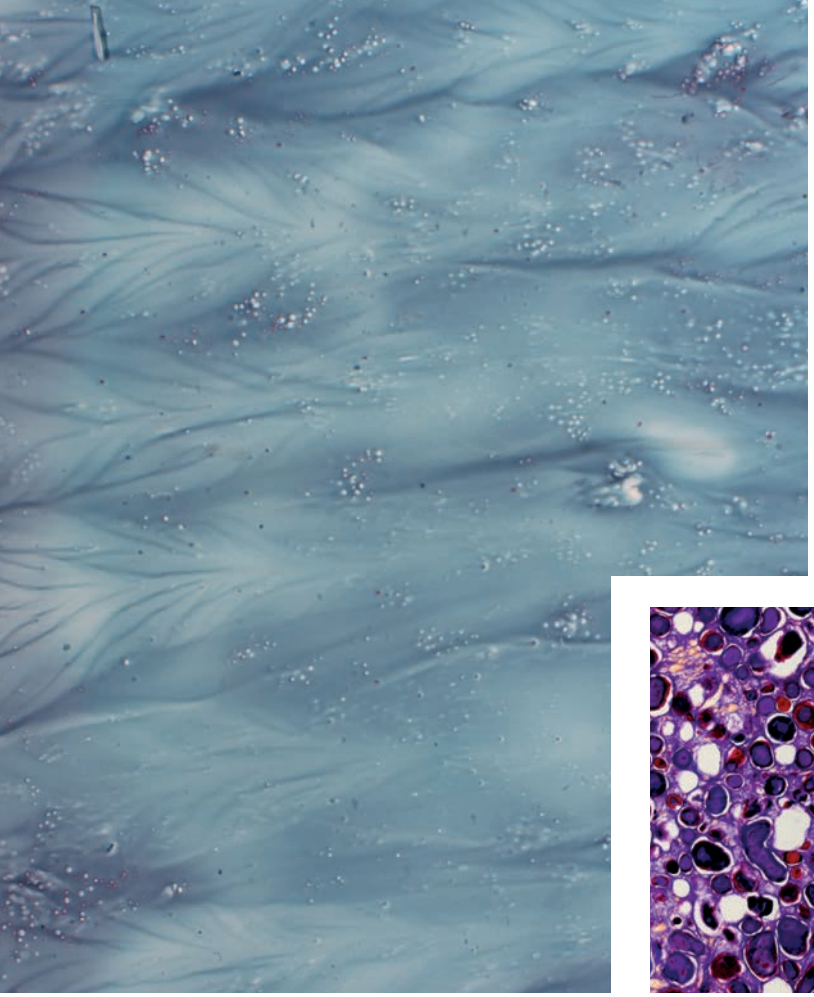
*Matthew Hanna and Liron Pantanowitz, University of Pittsburgh Medical Center, USA.*

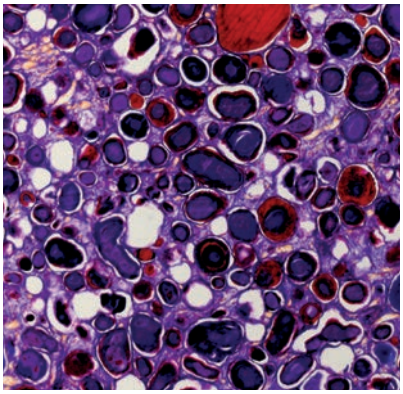


## RADIATION STRUCTURE OF THE TERMINAL ILEUM

The wall of the ileum in this traditional watercolor illustration is grossly thickened and the lumen narrowed, with a healthy section also displayed to show the difference (bottom right).

*Louise Hinman, artistamedico.com*







## IN A SFOG

Renal tubules in an acid-fuchsin orange G (SFOG) stain (top left) and thyroid colloid (middle).

*Simone Münst, University Hospital Basel, Switzerland.*

## CELLULARITY STUDY #3

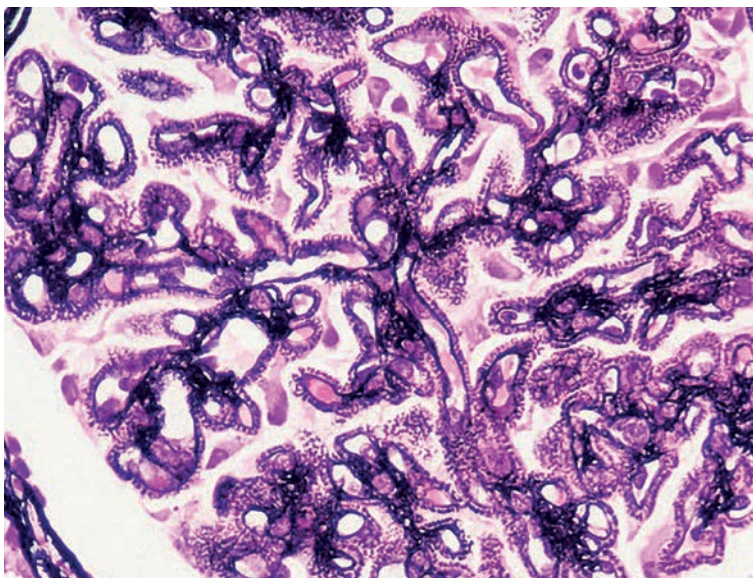
Acrylic on canvas (middle right).

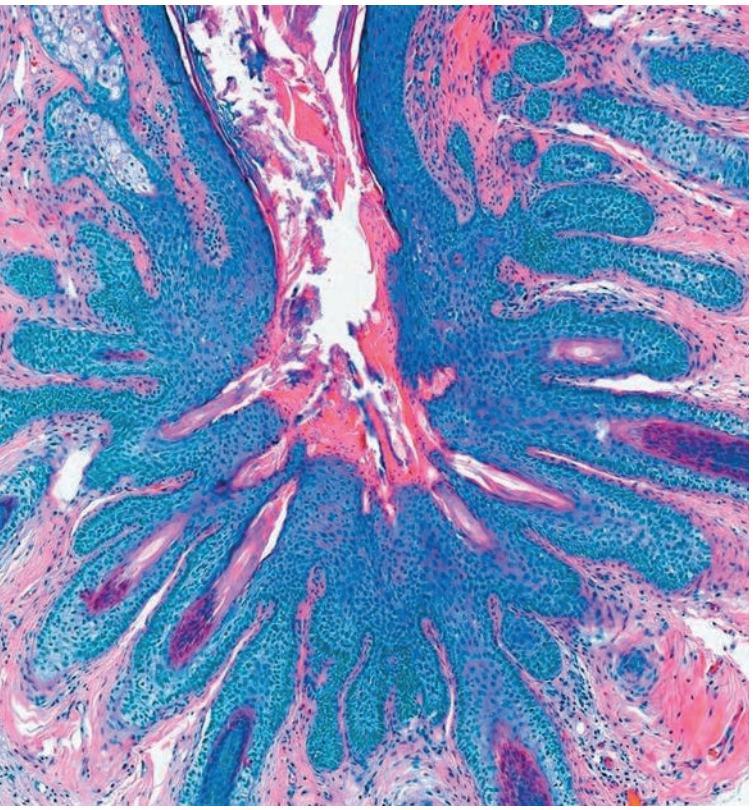
*Xiaoyin "Sara" Jiang, Duke University School of Medicine, USA.*

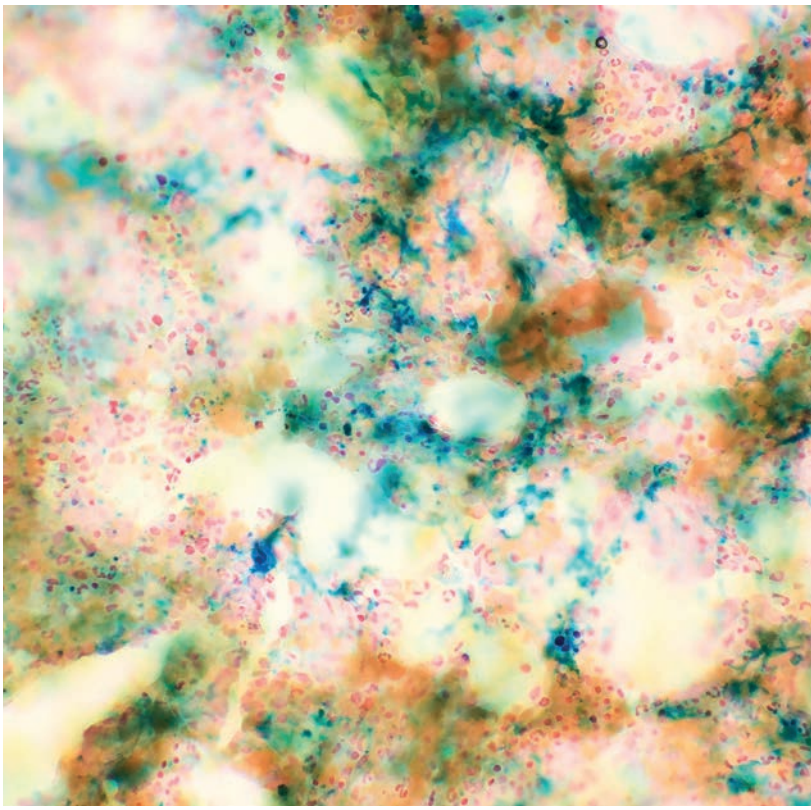
## INSIDE THE KIDNEY

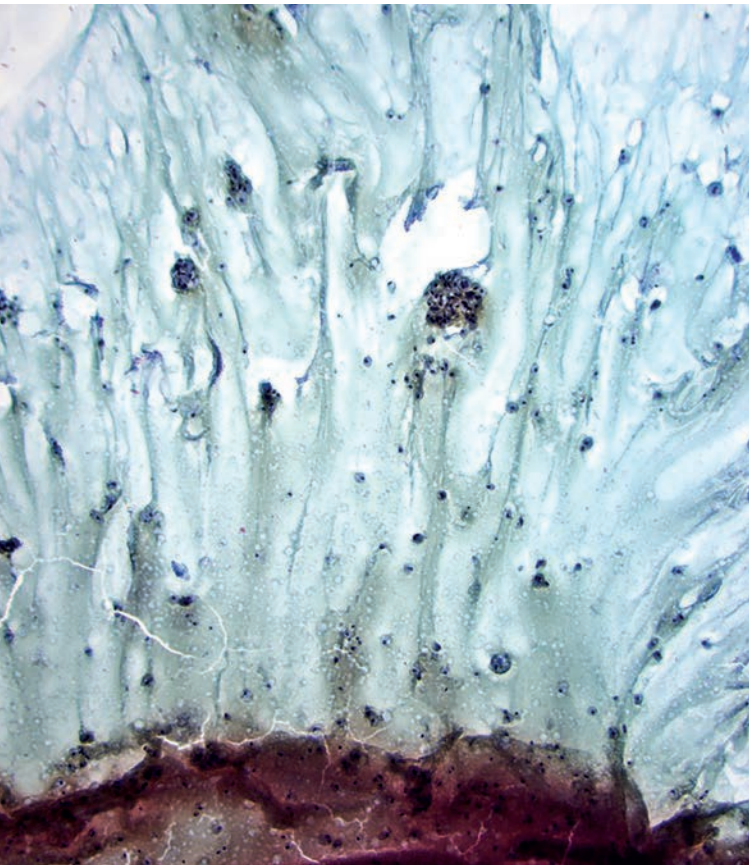
Membranous nephropathy (bottom right).

*Beth Braunhut, The University of Chicago, USA.*









## TRICHOFOLLICULOMA

A trichofolliculoma (benign skin tumor) is composed of primitive hair follicles radiating from a central pore. In this image (top left), the colors of the original H&E-stained tissue have been digitally altered for artistic effect.

## IRON MARROW

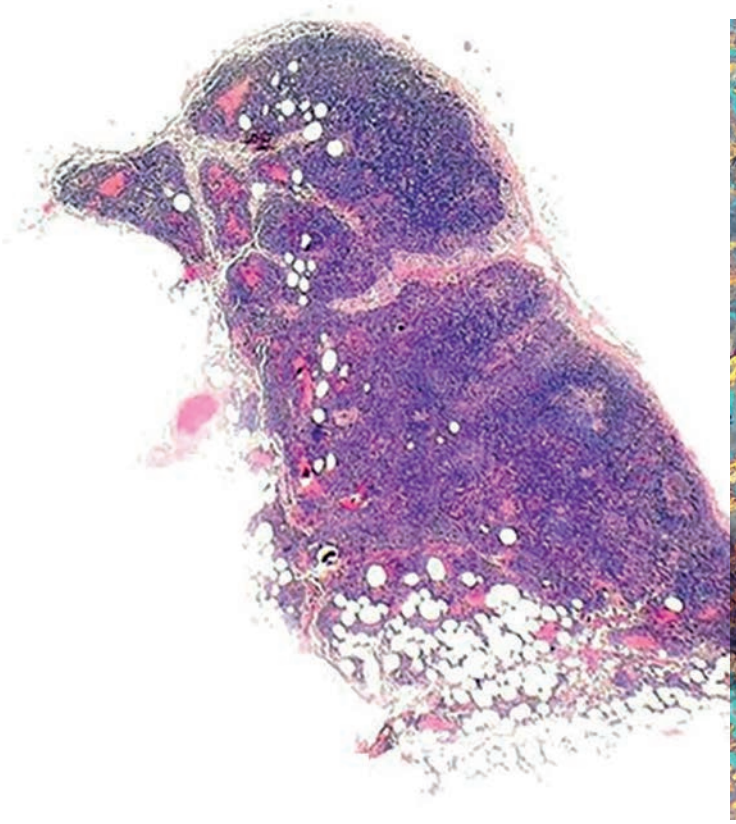
In this image of bone marrow (top right), the Prussian blue stain reveals the presence of iron.

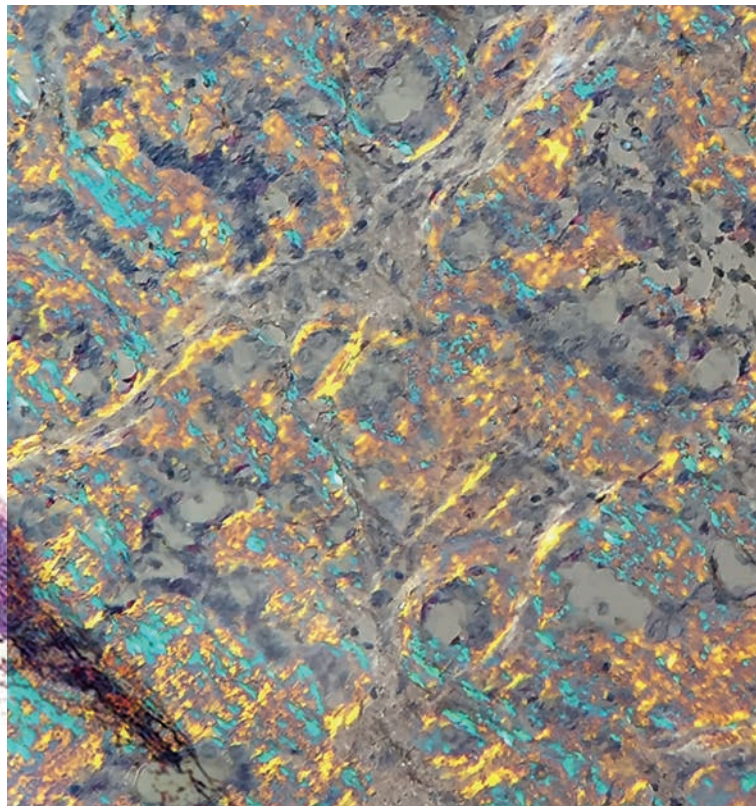
*Ziad El-Zaatari, Houston Methodist Hospital, USA*

## ABSTRACT PATHOLOGY

Cytology preparation of a nipple discharge (bottom).

*Darin L. Wolfe, Indiana Forensic & Surgical Pathology LLC, USA.*





## SENTINEL SPARROW

"Aviary" nice example of a benign lymph node (top left)!

*Katrina Collins, Hartford Hospital, USA.*

## KEEP ROTATING!

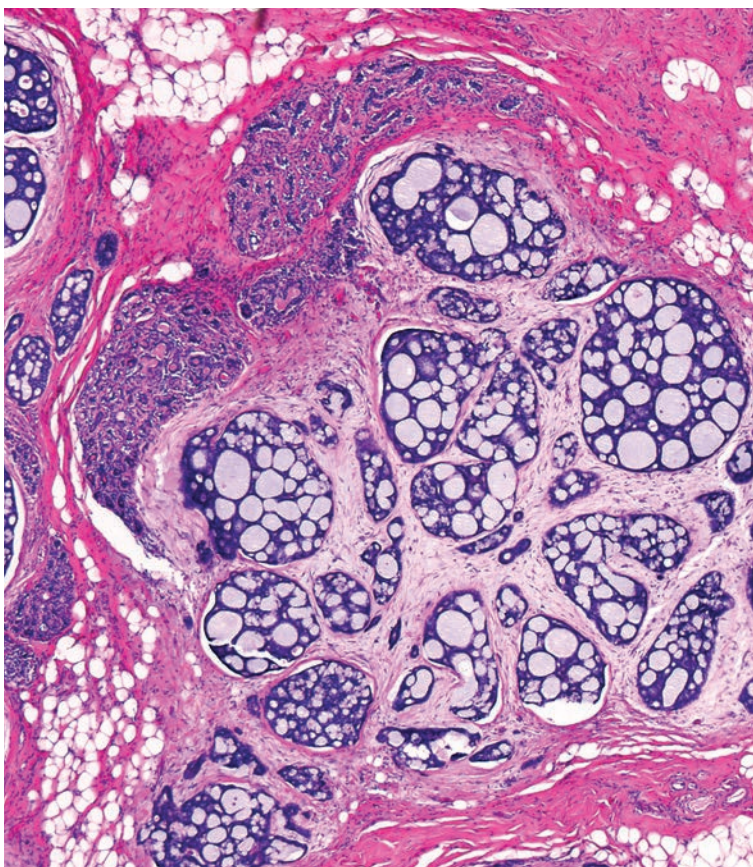
This is a high-power view of seminal vesicles showing amyloid depositions stained by Congo Red. Continue to play with your polarizer. You may encounter more beautiful colors than conventional apple-green birefringence!

*Woo Cheal Cho, @DrAldehyde*

## AN ACCURATE DIAGNOSIS

Adenoid cystic carcinoma of the breast.

*Dharam M. Ramnani, webpathology.com*



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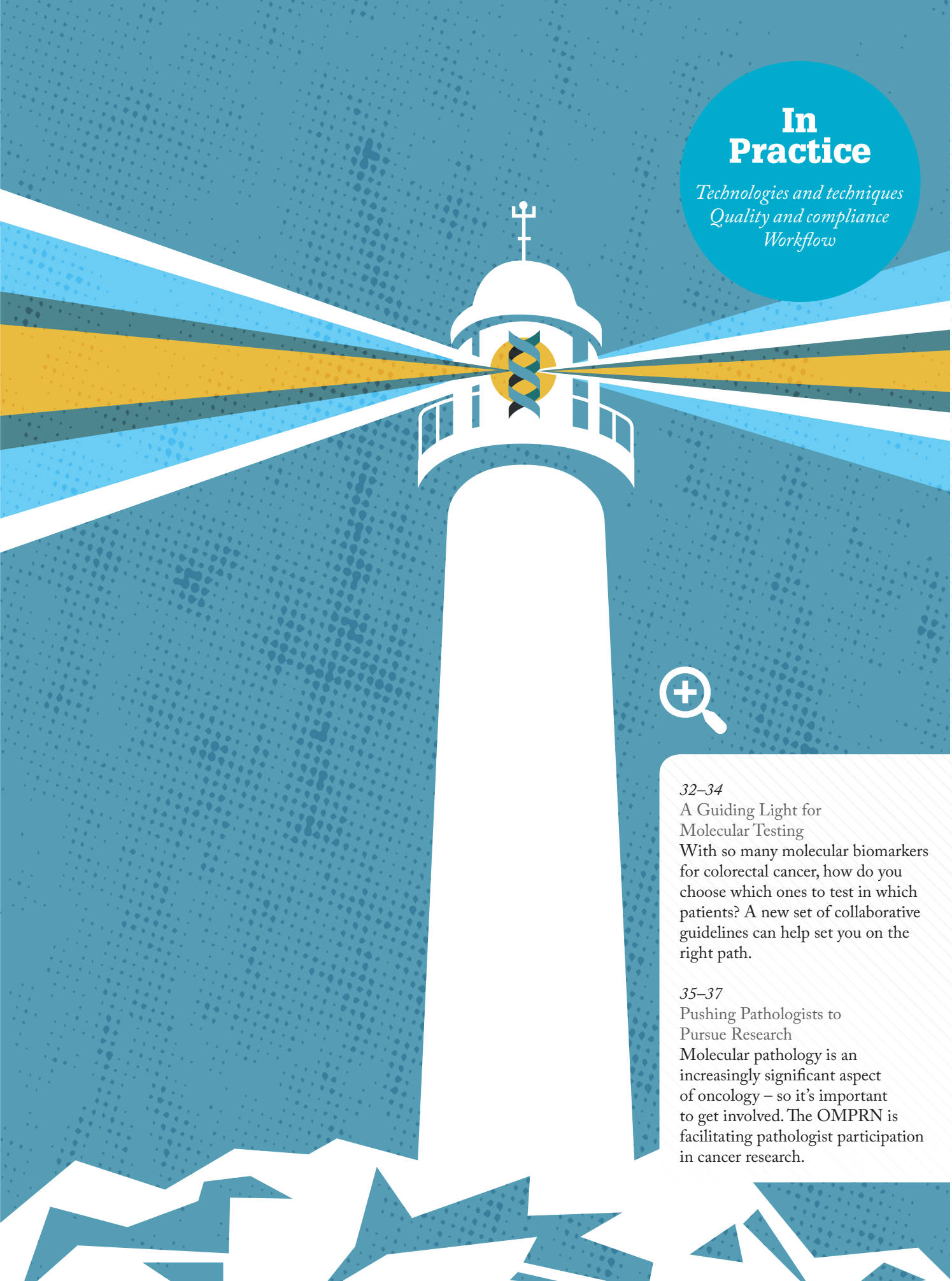


# In Practice

*Technologies and techniques*
*Quality and compliance*
*Workflow*

# A Guiding Light for Molecular Testing

## Four organizations collaborate on comprehensive guidelines for biomarker testing in colorectal cancer

*By Antonia R. Sepulveda*

It's clear that molecular diagnostics is a rapidly evolving field of medicine. It's also clear that, to be of use to pathologists, recommendations for the use of those molecular diagnostics must keep pace. Colorectal cancer (CRC) is the second leading cause of cancer-related death in both North America and Europe, and results from biomarker testing can help guide clinicians and oncologists on complex targeted therapy decisions to improve patient outcomes. But is current guidance sufficient?

Four professional organizations (AMP, ASCP, CAP, and ASCO) recognized a gap and decided to collaborate on biomarker testing recommendations that addressed new discoveries in the field. Together, we convened a multidisciplinary panel of experts – pathologists, oncologists, methodologists and more – to develop the guidelines and publish them simultaneously in each of the organizations' journals. Following the Institute of Medicine standards for guideline development, the expert panel conducted a systematic review of more than 4,000 articles in the field, evaluated the strength of the evidence, and compiled a list of guideline statements.

However, we wanted to make sure we weren't just handing down recommendations from on high, so we included patient advocates in the development process from the start and even held a public open comment period – during which we received hundreds of responses – to ensure that everyone's voices were heard. Those practices let us refine the guidelines to tackle issues specific to practicing pathologists and clinicians. And because we know that medical science is moving ahead in leaps and bounds, we've made sure to plan for regular updates, so that we can integrate new advances in molecular testing for clinical management of CRC as they happen.

The pivotal new guidelines from AMP, ASCP, CAP and ASCO address the testing of a wide range of molecular biomarkers in patients with early and advanced CRC to establish standard molecular biomarker

### *At a Glance*

- *Molecular diagnostics is a fast-moving field – existing guidelines are often piecemeal or don't cover the latest advances*
- *Colorectal cancer is one area where molecular biomarker testing is both necessary and valuable*
- *Four organizations – AMP, ASCP, CAP and ASCO – have collaborated on a new set of guidelines to cover predictive and prognostic biomarkers and molecular testing processes*
- *The new guidelines will be regularly reviewed to ensure they stay up to date with emerging biomarkers and testing technologies*

tests, guide targeted therapy decisions, and advance personalized care. And they're not just limited to recommendations regarding the indications to test for specific predictive or prognostic biomarker mutations; they also incorporate laboratory performance, reporting and proficiency testing of molecular assays.

Until now, we have predominantly performed molecular testing in CRC to assess the use of anti-EGFR monoclonal antibodies. Such targeted treatment requires knowledge of the mutational status of EGFR pathway genes as predictive biomarkers of disease response. And yet, prior to the new molecular testing recommendations, there was a lack of published comprehensive guidance on which gene mutations should be tested, what types of tissue samples to use, and whether the results provided predictive or prognostic information for CRC patients. To fill that gap, we developed a foundation of 21 guideline statements.

### What's in the guidelines?

Our 21 guideline statements (eight recommendations, 10 expert consensus opinions, and three "no recommendation") were established based on evidence from a systematic literature review. The guidelines support mutational testing for predictive biomarkers – genetic molecular biomarkers that forecast response to a specific therapy or treatment regimen. At this time, the predictive biomarkers that have strong evidence for testing in CRC patients are mutations in the *RAS* genes – *KRAS* and *NRAS* exons 2, 3 and 4. The first recommendation in the new guideline is to test for those mutations in patients who are candidates for anti-EGFR monoclonal antibody therapies, as noted earlier.

Not all biomarkers are predictive, though; some, like the *BRAF* V600E mutation, provide primarily prognostic information, and it's equally important to spot those alterations. For instance, we recommend testing for deficient DNA mismatch repair (dMMR) as it may have predictive value in some clinical settings. Although we recommend MMR testing for all CRC patients as a workup test to evaluate for possible Lynch syndrome, guidelines for its use as a predictive biomarker of response to therapy have not previously been reported. We believe it can provide prognostic information – patients with dMMR tumors tend to have improved survival rates over those with proficient tumors.

*"Markers like these have tremendous potential in clinical practice; the new guidelines tackle the question of how."*

Our recommendations also cover emerging biomarkers like microsatellite instability (MSI), as well as suggesting ways to streamline molecular testing processes for improved efficiency and better overall patient outcomes.

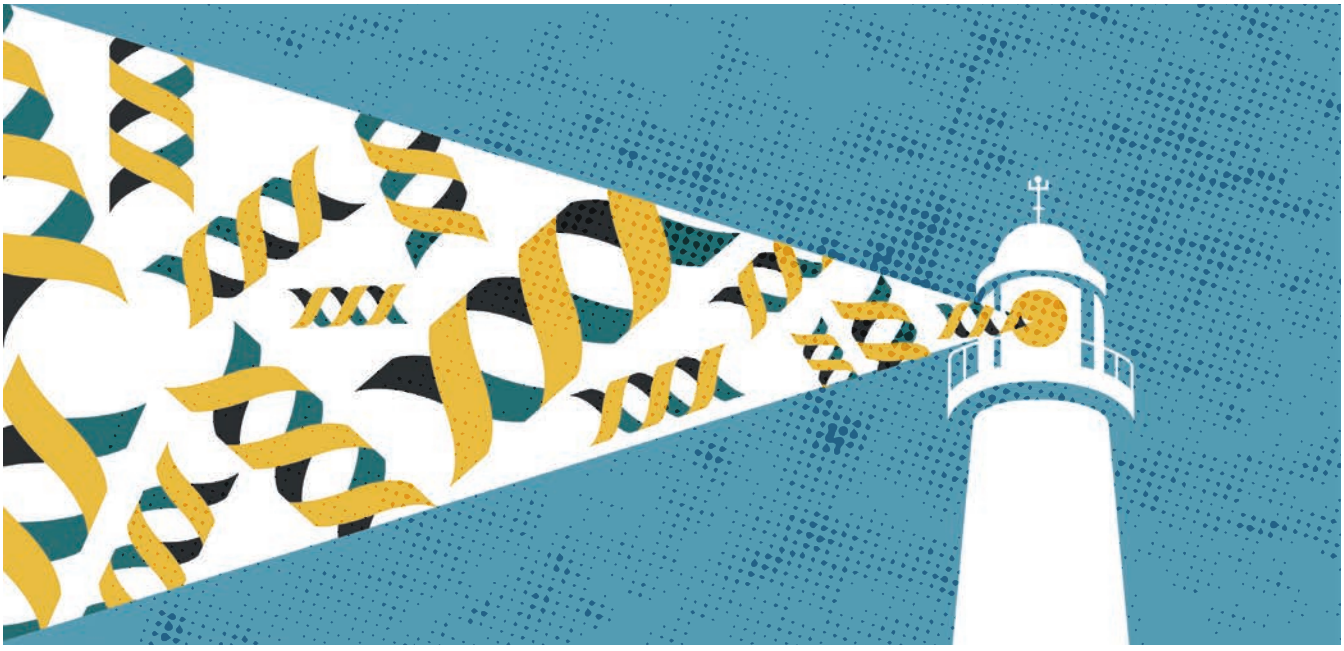
Indeed, MSI testing appears to be on its way to prominence. Why? Recent molecular biomarker data have shown the importance of MSI testing – a marker of dMMR – in selecting patients for immunotherapy (1). It's a brand-new indication for MSI testing in CRC, but a promising one. We know that alterations in a number of critical genes in CRC development and progression – such as dMMR and *BRAF*-activating mutations – can affect prognosis, as measured by several metrics of tumor progression or survival. It's clear that markers like these have tremendous potential in clinical practice; the new guidelines tackle the question of how.

### What does this mean for pathologists?

The post-genome era and the increasing emphasis on precision medicine are providing enormous amounts of new data. Promising molecular cancer biomarkers are emerging every day as potential diagnostic tools for identifying CRC patients, customizing their treatment, and forecasting its likelihood of success. But the speed at which the field is developing is a double-edged sword; it means that, every day, laboratories and regulatory agencies face the challenge of rapidly and efficiently providing new test results for the management of patients with cancer.

Laboratory testing of molecular biomarkers is a complex process. It involves selecting the right assay and the right type of specimen to test, timing the test appropriately, and minimizing turnaround time for testing results. We've learnt in recent years that we can effectively use a plethora of technical approaches as long as the sensitivity and specificity of the tests meet clinical needs. Earlier testing approaches focused on just one or a few testing targets, but nowadays, that isn't always enough. The current need for multiple molecular markers from potentially minute tumor samples is leading to greater use of gene panels – for instance, targeted next generation sequencing (NGS) panels – that can assay hundreds of genes and amplicons with known mutational hotspots.

We developed our recommendations to help doctors decide which molecular tests to order for patients with cancer. To get the most out of them, pathologists and oncologists need to work collaboratively to choose the testing approaches that best meet the clinical needs of their practice settings. If that happens, I anticipate that the comprehensive scope of the new guidelines will result in a more widespread adoption of standard approaches to



*"The comprehensive scope of the new guidelines will result in a more widespread adoption of standardized approaches to molecular testing."*

molecular testing – and, hopefully, enhance the treatment management and overall wellbeing of our patients.

### What's coming up next?

We've worked hard to establish these guidelines – but now, we need to make sure everyone knows about and understands them. To that end, we're launching an ongoing communication and information dissemination campaign to both professionals and the public. We hope it will increase awareness and assist in the integration of guideline recommendations into pathology, laboratory, and clinical practice.

We intend to review the existing guidelines every four years – or earlier, if any new research emerges that could potentially alter our original recommendations. We'll also continue to make additional recommendations to further streamline molecular testing processes and improve patient outcomes. Based on emerging testing technologies, I anticipate that more refined testing recommendations for specific platforms, such as NGS and liquid biopsy, will appear in future updates.

In terms of emerging clinical applications, it appears that we may soon need recommendations regarding testing to identify CRC patients who are likely to respond to immunotherapy blockade. To determine for sure what needs to be done, though, we'll need to wait for sufficient published evidence – which means that, at the moment, it's difficult to predict a timeline.

For some time, we've had an array of recommendations that cover the application of individual molecular biomarkers to CRC. What's different about our guideline is the fact that it's a single, comprehensive unit. It fills the need for an overarching set of recommendations that span the breadth of our current knowledge. My hope is that, as the application of these new recommendations becomes widespread, we'll start to see better outcomes for all of our colorectal cancer patients.

*Antonia R. Sepulveda is Project Co-Chair on behalf of the Association for Molecular Pathology, and is Professor of Pathology and Cell Biology and Vice Chair for Translational Research at Columbia University, New York, USA.*

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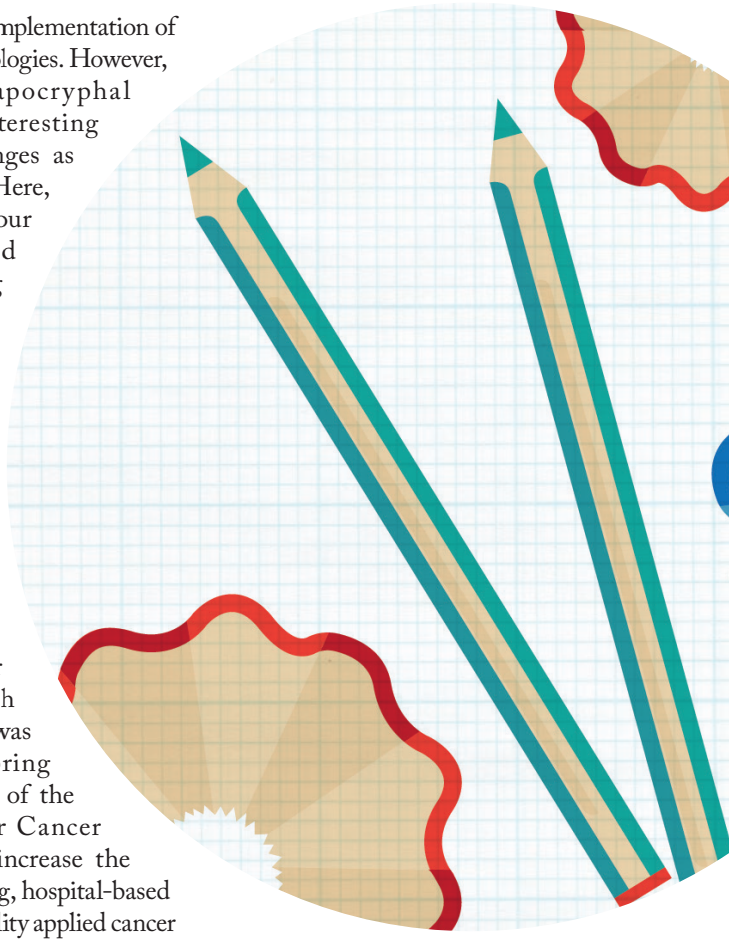
# Pushing Pathologists to Pursue Research

## How the OMPRN is changing the face of Canadian cancer research by boosting molecular pathology education

*By David LeBrun and John Bartlett*

And we certainly do live in interesting times. The explosive expansion of our understanding of cancer biology, coupled with the availability of hundreds of "targeted" cancer drugs, has created enormous excitement amongst oncologists, researchers and patients. As the key medical professionals tasked with extracting clinically actionable information from diagnostic tissue samples, pathologists find themselves with an enviable opportunity to improve the lives of cancer patients by expediting the clinical implementation of new concepts and technologies. However, as implied by the apocryphal Confucian curse, "interesting times" present challenges as well as opportunities. Here, we discuss some of our own tried and tested approaches for ensuring that both new and existing pathologists grow familiar with these novel concepts and technologies, allowing them to expand their molecular repertoire.



### *At a Glance*

- *Our understanding of cancer biology is expanding, as is the availability of targeted drugs – but these rapid developments can present a steep learning curve for pathologists*
- *The OMPRN was established to increase pathologist involvement in the latest cancer research, and to promote education in the latest techniques and concepts*
- *Effective methods include targeting funding, research partnerships, workshops, and collaborating to update educational curricula*
- *Many still consider molecular techniques supplementary, but the oncology landscape is changing fast, and boundaries between disciplines are becoming ever more blurred*

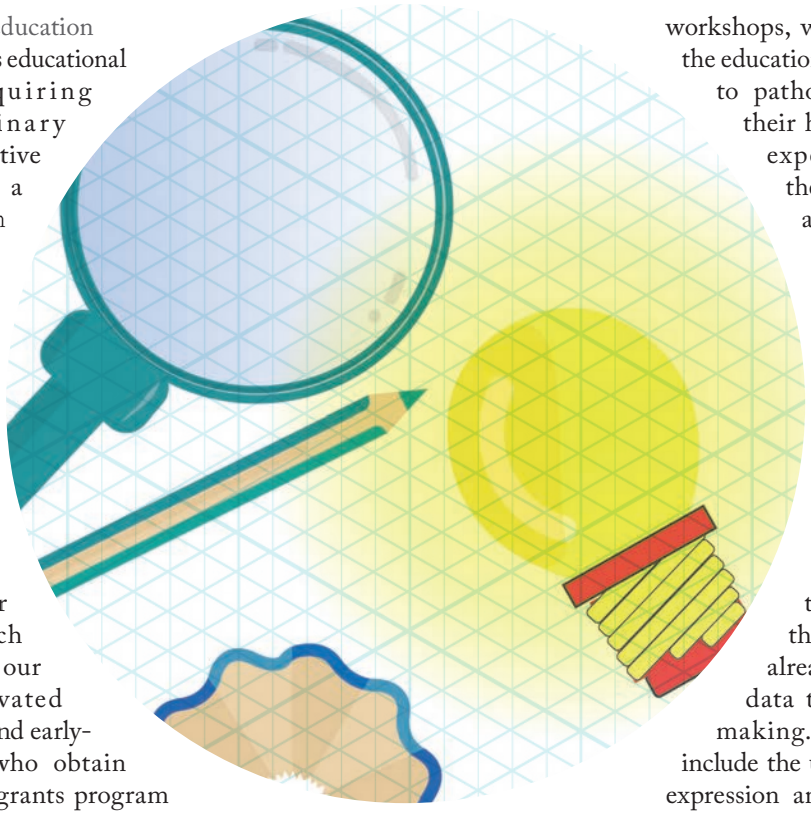
### Connecting and coordinating

The Ontario Molecular Pathology Research Network (OMPRN) was established in the spring of 2016 as a program of the Ontario Institute for Cancer Research (OICR) to increase the involvement of practicing, hospital-based pathologists in high-quality applied cancer research. This investment of almost CA$4 million over four years reflects recognition by the OICR of the important role that pathologists can – and should – play in connecting and coordinating diverse cancer research activities across the province. For example, pathologists can contribute to the design and undertaking of biomarker research associated with clinical trials and, importantly, pursue their own research questions in a manner informed and augmented by transdisciplinary collaboration. At the same time, they can help basic and clinical scientists gain appropriate access to high-quality biospecimens.

In deciding how to deploy resources to maximal effect, organizations must consider the multiple roadblocks that prevent practicing pathologists from becoming fully involved drivers of research. These include limited time available for research pursuits, limited training in research design or techniques, limited access to specialized technical resources, and the extremely competitive nature of the current research funding environment. With these issues in mind, we elected to provide targeted funding for pathologist-led, transdisciplinary research projects. In addition, because education represents a cost-effective and feasible means of guiding and encouraging the adoption of new concepts and methods in research and practice, we also prioritized the education of pathologists and pathology trainees.

## Education, education, education

One way we address this educational priority is by requiring both transdisciplinary collaboration and active participation by a pathology trainee in the research projects we fund. That helps us to broaden the experience and career options of trainees and incentivize collaboration across interdisciplinary boundaries. The fact that trainees self-select or are nominated for involvement in research projects helps direct our investment to motivated individuals. Trainees and early-career pathologists who obtain funding through the grants program also automatically join OICR's Pathology Club, whose members meet quarterly to report on the progress of their projects and obtain advice from peers and experienced researchers. The Pathology Club complements the grants program and contributes to the development of a peer group of motivated young pathology investigators.



*"Our goal is to work with the existing governing bodies to ensure that all "freshly-minted" pathologists can practice competently in the molecular era."*

The OMPRN also facilitates access to a series of workshops that help pathologists and trainees acquire skills applicable to pathologist-driven research. Workshops that have already taken place or will take place in the near future include sessions on gene expression profiling, fluorescence in situ hybridization, PCR-based analyses, next generation DNA sequencing, bioinformatics, biostatistics, computer-assisted image analysis, and the design and conduct of clinical trials. We develop and host some workshops in-house, and in other instances we encourage pathologists to attend workshops run by other organizations (for which we cover the associated costs). In considering the content of these workshops, we intend to complement the educational opportunities available to pathologists and trainees at their home institutions. In our experience, initiatives like these can help both trainees and working pathologists to update their knowledge and stay current.

## The rise of molecular methods

Until recently, Canadian pathology training programs have devoted a relatively small portion of their curricula to molecular concepts and techniques. This is despite the fact that clinicians are already relying on molecular data to guide their decision-making. Well-known examples include the use of mRNA-based gene expression analyses and EGFR gene sequencing in breast cancer and non-small cell lung cancer, respectively. Mid-career pathologists are already adapting their practices to incorporate molecular methods – so pathology training programs, both in Canada and around the world, must prepare their trainees accordingly.

Collaboration is the key. We are working with pathology representatives at the Royal College of Physicians and Surgeons of Canada (RCPSC) to inform the evolution of pathology training curricula not just in Ontario, but across Canada. Our goal is to work with the existing governing bodies to ensure that all "freshly-minted" pathologists can practice competently in the molecular era.

With this in mind, an important near-term challenge for all educators is to decide which molecular concepts and competencies are likely to be of most use to practicing pathologists – and to ensure that they are introduced during training.

Worthy candidates include (1):

- theoretical knowledge about genomics, epigenetics, gene regulation, protein structure and function,
- applied knowledge pertaining to the indications for molecular testing,
- pre-analytical variables that may affect testing results, and
- the interpretation and clinical implications of results.

Once identified, these priorities can inform recommendations for modifications to residency training programs. We expect that revised residency curricula will use a combination of didactic instruction, "on-the-job" training during subspecialty rotations, independent reading or computer-assisted instruction, and, when specialized resources are required, off-site workshops sponsored by the OMPRN or others. We began this process by convening an Education Committee that includes researchers in pathology and diagnostic molecular genetics. The Committee reviewed current literature, considered what Canadian residency training programs are already doing to incorporate molecular content into their curricula, and generated a draft list of molecular competencies. Then, we initiated a dialogue with the RCPSC Specialty Committee in Anatomical Pathology. At the moment, we are looking forward to working with them, pathologist educators and other stakeholders in the Canadian oncology community to modernize pathology residency training.

## Evolve to survive

Many people equate the medical specialty of pathology exclusively with histopathology. As our colleagues will know, this is a simplistic view – it ignores the important evolutionary changes of the last several decades that have required pathologists to incorporate molecular assays into routine diagnostic practice. However, it remains largely true that pathologists themselves still tend to consider conventional histology their primary diagnostic approach and typically refer to molecular techniques as ancillary at best.

But the technical and intellectual landscape in oncology is changing rapidly. To stay current – and to improve the experiences and outcomes of patients – pathologists, laboratory-based clinical scientists, clinical oncologists and colleagues must work together seamlessly, and perhaps even reconsider the boundaries that have historically defined their respective disciplines. We hope that our own efforts in this area can provide inspiration to other pathology organizations seeking to embrace the latest developments in molecular science.

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*John Bartlett is Education Lead of the Ontario Molecular Pathology Research Network and Program Director of Diagnostic Development at the Ontario Institute for Cancer Research, Canada.*

*We thank Rebecca Tamarchak for her insightful comments on the manuscript.*

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# NextGen

*Research advances*
*New technologies*
*Future practice*

# Slides in the Machine

## Digital pathology is the future of storing and sharing images of tissue – and combining it with deep learning could further transform the field

*By David West, Jr. and Hunter Jackson*

Artificial intelligence in healthcare is gaining ground in areas ranging from patient care to diagnosis, data management, and many others. And although we're still a long way from reaching full automation, pathology is ripe for major disruption.

Gone are the days of having cabinets full of slides that are difficult to share and only viewable by squinting down a microscope. Pathologists can now examine tissue on their computers from anywhere in the world via whole slide imaging – and, with the help of computational pathology software powered by intelligent machine learning, will have new, precise information at their fingertips. The digital revolution is already underway.

### *At a Glance*

- *In the past, digital pathology has been held back by software and storage issues – but advances such as cloud computing are breaking down these barriers*
- *Whole slide imaging can improve efficiency, increase automation, and improve collaboration between institutions all over the world*
- *Machine-learning-driven "computational pathology" may also result in new biomarkers, workflow improvements, and image-based diagnostics, providing pathologists with new tools for diagnosing disease*
- *FDA approval of the first WSI system is a turning point for the technology, and soon "digital pathology" may instead simply be "pathology"*

### From slides to files

Today, digital whole slide imaging (WSI) allows the capture of the entire tissue sample on a slide, as opposed to using a microscope camera attachment to capture a field of view. A modern scanner takes between 30 seconds and two minutes to capture a slide, usually at a magnification of 20x or 40x. The resulting image files are very large, allowing us to capture a great deal of data, but typically require specialist software to view and sophisticated IT solutions for storage. It's not an incredibly new system (it has existed for research and education uses for some time) but we can expect to see increased adoption of WSI as technology advances and as storage issues are addressed. Progress will result in a new realm of possibilities for tools that enhance how pathologists review cases.

### On efficiency and algorithms

Simply switching to a digital workflow has been shown to increase efficiency by 10 to 15 percent (1). But beyond these baseline improvements lie exciting and transformational benefits. Once we're in the "digital realm" and dealing with pixels rather than glass, we open up two key possibilities. Firstly, distance no longer matters. Sending slides between two institutions on different sides of the world becomes much easier, opening up new telepathology possibilities, helping pathology departments and private labs to

grow, and improving access to subspecialty experts for patients in remote areas.

Secondly, image analysis algorithms will be able to operate on the images. These are already used for automated or semi-automated immunohistochemistry quantification, helping to drive standardization and speed of analysis – and new methods are in development to augment H&E. The area could play a profound role in reducing inter-observer variability for many cancers, and in driving faster, more precise quantitative workflows.

## Fighting preconceptions

There's certainly some resistance to going digital, but most are rooted in challenges that are easy to overcome. One of those challenges is resistance to cultural change. We find the 4best way to address resistance is to give pathologists the opportunity to try it firsthand. Seeing is believing. It's common for a pathologist to have negative preconceived notions based on experience with older digital pathology systems or just the "idea" of digital in general, so it's especially rewarding to see a pathologist's eyes light up when they see the quality of images, what quantitative tools can do today, and how simple collaboration with colleagues becomes. These systems can increase the efficiency of pathologists by automating monotonous tasks, such as identifying mitotic cells or screening benign tissue and identifying "cancer hotspots." Pathologists can then focus their time on making informed diagnoses.

Another concern is image quality and throughput – and a year or two ago, this was indeed a larger concern than it is now. But these issues are improving every year, digital storage costs are going down, and the first WSI system was just approved by the FDA for clinical use – we are truly at a turning point.

## Embracing AI

As mentioned above, another great advantage to digital slide imaging is the potential for automated analysis. Deep learning is a class of machine learning algorithms that have been around for decades, with some major breakthroughs in the last few years. Newer learning techniques can be used to allow image and pattern recognition. The results so far have been remarkable (2), and are generating excitement in many fields, especially medical imaging.

There is now a well-timed confluence of events; the digitization of pathology so far is already generating lots of data, new methods are becoming available to technologists, and enormous amounts of computing resources are being made available via the cloud at low cost. All of this means that we can start training intelligent algorithms to recognize broad or specific patterns on a whole slide image, and translate features evident in the tissue into prediction (such as metastasis and recurrence) and classification (staging, grading, and differential diagnosis). This enables the creation of predictive biomarkers based on precise measurements of histological patterns, providing pathologists with new tools to answer questions about a given patient's disease. This is especially useful where molecular tests fall short – image-based assays could be part of a portfolio of tests in a pathologist's precision medicine arsenal. Scanned slides can be used to train deep neural networks to learn how the cellular morphology reveals genetic and epigenetic changes in the tissue. In some ways, it's still early days, but this is already starting to be used in cutting-edge laboratories, and deep learning is likely to have a broad range of applications within pathology.

For example, cancer is a system and should be evaluated on a spectrum, so pathologists need many tools to uncover both molecular and morphological changes in the tissue to place a specific case of cancer on that spectrum. Many genetic tests can help genotype a patient's cancer, which can help inform therapeutic options. However, to qualify for certain genetic tests, you must meet rigorous requirements, including overexpression of certain proteins and evident genetic alterations. With the advent of image-based diagnostics, it may be possible to expand the patient population that is eligible for these tests, informing precise treatment plans.

## Is the future in the computer lab?

FDA approval of the first WSI system is exciting news, especially as many people thought it might never come. More vendors will likely follow, and in a few years, "digital pathology" may simply be "pathology." It's not going to happen overnight, but quantitative, computational-driven workflows will be one of the key drivers for market adoption, and laboratories that resist this change will likely fall behind more forward-thinking peers. As more slides are scanned and more data becomes available, we'll likely see algorithms seep into the workflows of pathology labs. Early adopters are already using these algorithms to augment their work. In the next ten years, we anticipate that much of the professional component of pathology will be divorced from the physical laboratory, with human pathologists working in software driven "labs."

*David West, Jr., is President and CEO of Proscia Inc.*

*Hunter Jackson is is VP Research at Proscia Inc.*

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# Liquid Assets

## Is ctDNA analysis the future of cancer pathology? We speak with two experts at the leading edge of liquid biopsy

*By Roisin McGuigan*

Liquid biopsies are becoming increasingly commonplace and, in some cases, are now in clinical use. In cancer, they allow tumor DNA to be sequenced and examined without the need for invasive and potentially risky tissue biopsies. We spoke to two researchers pioneering myriad potential applications of liquid biopsies and circulating tumor DNA (ctDNA). Lao Saal is working to detect occult metastases by identifying and tracking chromosomal rearrangements. Isaac Garcia-Murillas has developed a novel approach to identify the risk of relapse among patients with early-stage breast cancer.

### What drew you to cancer research?

I*saac Garcia-Murillas:* I'm a molecular biologist by training, and I followed a standard career path – a PhD followed by a postdoctoral fellowship. Much of my work had been on intracellular signaling, but when I joined the lab of oncologist Nick Turner, we started to focus on ctDNA projects. My work took on a whole new meaning when it came to cancer – and to translating our research to the clinic.

*Lao Saal*: It goes back almost 20 years, to my days at the National Human Genome Institute at the US National Institutes of Health. I'd been studying pediatric cancer genomics and started thinking about ways to apply innovative methods to better understand tumor biology, improve diagnostics, and ultimately deliver precision therapies. After finishing my MD and PhD degrees in New York and gaining some postdoctoral experience, I came to Sweden in 2009 and started my own research group at Lund University.

### What are you currently working on?

*IGM*: My work mostly involves the use of plasma or serum separated from blood, and I am particularly interested in ctDNA and its use as a biomarker for the detection of Minimal Residual Disease (MRD) following neoadjuvant chemotherapy and/or surgery. I'm also studying the use of ctDNA as a biomarker for detection of resistance to targeted therapies. My work is mostly in breast cancer, but I have interest in other solid tumors as well.

*LS:* Liquid biopsy for cancer is an extremely promising and exciting field, and I believe it will revolutionize how we diagnose, monitor, and treat cancer patients. Over the last couple of years, we have continued working on new technologies for ultrasensitive quantification of ctDNA, and now have several innovative approaches that we, together with partners and collaborators, are applying to measure ctDNA in patients with non-small cell lung cancer, melanoma, breast cancer, acute myeloid leukemia, and ovarian cancer, among others. These technologies are now part of SAGA Diagnostics AB, our cancer genomics company that focuses on molecular diagnostics and cancer liquid biopsies. One of our approaches marries NGS with droplet digital PCR (ddPCR) to measure chromosomal rearrangements as exquisitely specific truncal clonal markers in ctDNA and is particularly suited for patient monitoring and detection of MRD or relapse. We have another method that largely mutes base misincorporation errors – an issue that plagues all methods employing polymerases, such as NGS, qPCR, and ddPCR. With this proprietary method we can achieve an unparalleled limit of detection of one mutant molecule in a background of 100,000 wild-type molecules.

> *"I believe liquid biopsy will revolutionize how we diagnose, monitor, and treat cancer patients."*

### Liquid biopsy hasn't just seen success in cancer…

*LS:* Absolutely – I would even go as far as to say that it's probably gone further into the clinic in the field of noninvasive prenatal diagnostics. However, cancer is very close behind, and there's a lot of research and work going on. I think liquid biopsies will start to enter the clinical arena much more in the next five years.

There is also some really interesting work in infectious disease and other fields, such as transplant medicine – for example, to monitor whether a transplanted organ is showing signs of rejection.

### *At a Glance*

- *Liquid biopsy and circulating tumor DNA are both gaining ground in academic and clinical settings*
- *Two scientists at the forefront of developing the latest techniques discuss opportunities and pitfalls*
- *More standardization is needed for the approaches to become truly commonplace – and pathologists will need to embrace molecular methods*
- *Looking further into the future, liquid biopsy could become as routine and commonplace as diabetes blood sugar monitoring is today*

The benefits are clear – but what are the drawbacks?

*IGM:* In my opinion, the major pitfalls come from improper sample acquisition, preparation and analysis. The level of sensitivity required by these non-invasive techniques might be hampered by any of the above, rendering results invalid. I also think that liquid biopsies will remain a companion to tissue biopsies, at least for the time being, as there are many questions – including histological and pathological ones – that we cannot currently answer with these techniques.

To fully incorporate liquid biopsies into the management of patients, we need to further investigate their validity through more clinical trials. This will allow a better understanding of the advantages and limitations of these new approaches. I am unable to speculate on timelines, but I have no doubt that through our work and that of our colleagues in the field we will be in a position to start fully incorporating these techniques relatively soon.

*LS:* For ctDNA analysis to be more useful in earlier stages of cancer or to detect resistance mutations at the earliest moment, we need a method with exceedingly high sensitivity and specificity. That's one reason we are pushing hard to innovate and improve liquid biopsy technologies. There are also a number of areas where additional knowledge will help us make better use of liquid biopsy. For example, for each cancer type it's not entirely clear what the optimal schedule for sample collection is, especially in relation to the timing of therapies. We know that ctDNA quantity is closely correlated to tumor burden and that it is a powerful prognostic marker, but how the slope of the curve between ctDNA and tumor volume varies between cancer types is still being figured out. Given the wide successes of immunotherapies, it will be interesting to see how ctDNA analysis may be used to predict response. Depending on the cancer type, different liquid biopsy sources may be more informative, whether it be blood plasma, urine, cerebrospinal fluid, sputum, or other secretions. As a field, we also need to establish clear standards for reporting, so that studies can be compared more easily.

In some cases, ctDNA liquid biopsies are already entering the clinic. Today, this is most notable in advanced non-small cell lung cancer, and detecting the "druggable" mutations in the *EGFR* gene, such as L868R, the T790M resistance mutation, and the exon 19 deletions. Gradually, as the evidence accumulates, liquid biopsies will be increasingly used in the clinic

and will become rather commonplace. I think of ctDNA as a special kind of cancer biomarker – a biomarker that carries certain biological properties that are inherent and independent of the technology used to detect and quantify it.

How do you think the roles of pathologists and laboratory medicine professionals will change with the rise of liquid biopsy and similar techniques?

*LS*: I think pathologists will transition to molecular pathology more and more, and be increasingly involved in liquid biopsy diagnostics. As with any new diagnostic approach, there will be a learning curve and a great deal of educational effort will be needed. Many of the technologies could be deployed in local hospitals, given that NGS, qPCR, and ddPCR instruments are increasingly found in many clinical labs. However, the greatest cost-effectiveness and efficiencies of scale will only be possible at very large institutions or at centralized regional service laboratories.

What are the challenges when using ctDNA?

*IGM*: There are always challenges with any project – most of them technical. There are also biological challenges, and sometimes recruiting patients to provide samples can be difficult too. In our research into detecting aberrations in ctDNA, the main technical challenge is identifying the ctDNA, as the fraction of tumor DNA present in the total circulating DNA is very small. In the case of MRD detection, the loss of materials when extracting ctDNA from plasma was also an issue, as this obviously affects the amount of material available for testing.

Do you have any tips for those looking to implement liquid biopsy?

*IGM:* Researchers should always take extra care when obtaining and processing samples for molecular analysis – and that's particularly true for liquid biopsies, which are prone to contamination from other more abundant material coming from the patient. But I personally think that anybody trained in molecular biology techniques and handling human samples should be more than capable of working with liquid biopsies.

*LS:* People interested in implementing liquid biopsy in their own laboratories should carefully review the various options available, from the resource-intensive NGS approaches to the faster, more sensitive, but less broad qPCR and digital PCR approaches. The dynamic range of ctDNA is extremely wide: anywhere from just one mutant molecule to many tens of thousands may be floating in a 1 mL plasma sample, therefore assay characteristics like sensitivity and specificity are critical, as is the assay's lower limit of detection. As repeat testing is one of the benefits of liquid biopsies, consideration should be paid to the intended use and cost-effectiveness of the test. In some instances, a wide panel-based approach may be appropriate, whereas in other situations a focused test for just the clinically actionable mutations may be all that is required.

*"As with any new diagnostic approach, there will be a learning curve and a great deal of educational effort will be needed."*

What are your predictions for the future of ctDNA analysis?

*IGM*: The real revolution that is still to come is the so-called 100-dollar genome. I think that's going to revolutionize not only cancer research, but genomics research in general. New techniques arrive every few years, but my feeling is we will know more about the genomics of the individual at a larger level, for a much lower price. And this will also raise a lot of ethical and data protection questions…

*LS:* The fact that a ctDNA sample is so easy to collect, can be used to interrogate all tumor deposits simultaneously, and even carries quantitative prognostic and treatment-predictive molecular information, makes it almost the perfect cancer biomarker. Next, we have to think in terms of large populations, and think about how our research could benefit everyone, regardless of social status or race. At the moment, I think studies have been slightly biased towards certain populations. But as health professionals and researchers we have the duty to make our work available to everybody.

I believe that in the more distant future, ctDNA will be as commonplace and simple as diabetes blood sugar monitoring is today. I think it could also have a role in screening for cancer in apparently healthy people – or perhaps within groups that are considered high risk for certain cancers. The future of this area is really exciting, and it's great to be driving things forward, and getting closer to delivering precision medicine to cancer patients. Liquid biopsies and ctDNA give us the potential to ensure that people aren't burdened by therapies they don't need and instead get access to the therapies they do need – with the ultimate end goal of extending both life and quality of life.

*Lao Saal is Associate Professor and Head of the Translational Oncogenomics Unit at Lund University, Sweden. He is also founder and CEO of SAGA Diagnostics.*

*Isaac Garcia-Murillas is a Senior Scientific Officer at the Institute of Cancer Research in London, UK.*

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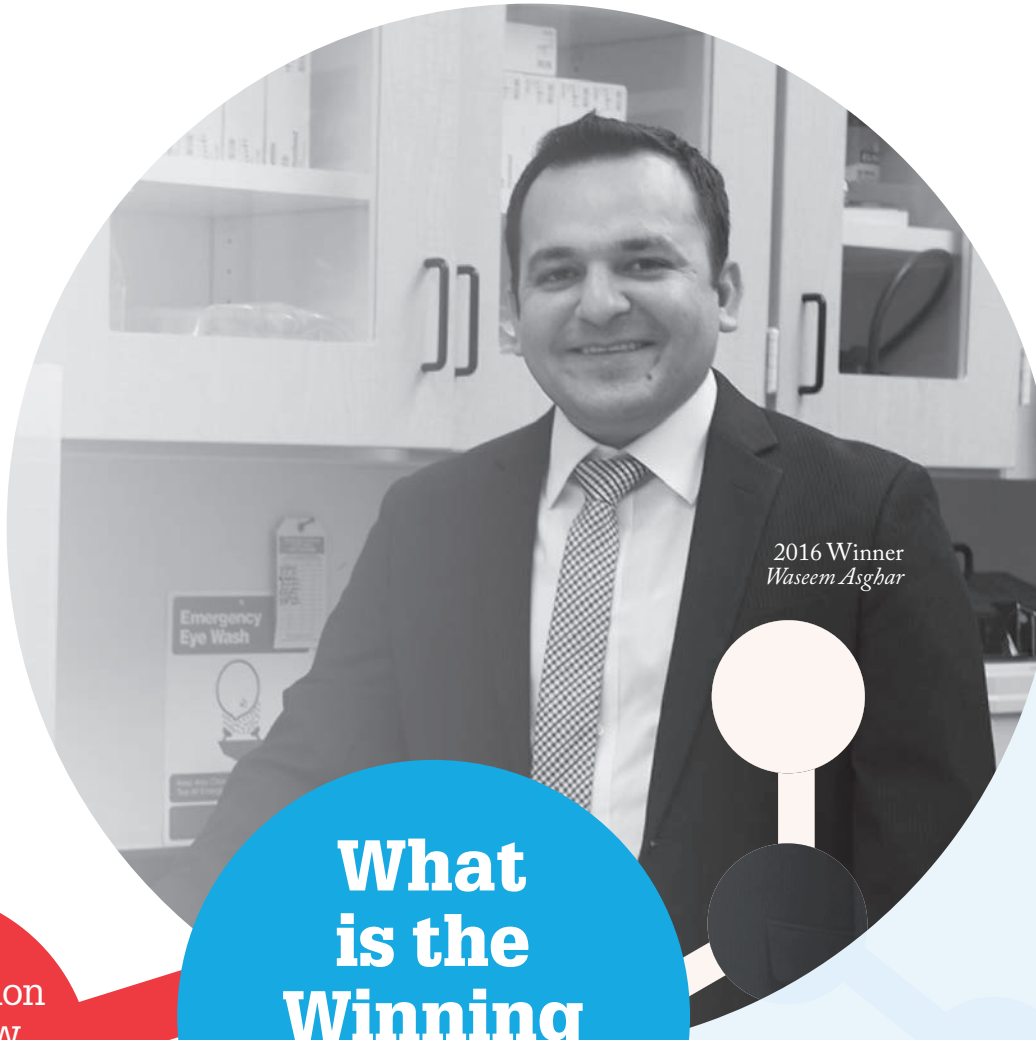


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# Solving the Mystery of Medical Mistakes

**Why do medical errors occur? A better understanding of the brain mechanisms involved in diagnosis can help answer the question – and avoid future issues**

*By Michael Schubert, based on an interview with Marcio Melo*

Diagnostic error. It's something we're all aware of, but hesitant to discuss (1,2). Whether we talk about it or not, though, there's one obvious question to address: how can we reduce the rate at which it occurs? Perhaps the key lies in understanding why we might make mistakes during diagnosis.

## *At a Glance*

- *A new functional magnetic resonance imaging study shows that diagnosing disease engages the same brain mechanisms used to identify and name everyday objects*
- *Highly diagnostic information at the beginning of assessment may predispose to premature diagnostic closure, an important cause of error*
- *A dramatic shift in brain activity between making and speaking decisions suggests that doctors need to hear themselves, either silently or aloud, to become aware of their diagnostic decisions*
- *The above holds true for simple diagnoses; the next step is to investigate the brain's activity during more complex decision-making processes*

What takes place in the brain between seeing a patient or sample and reaching a conclusion? Until now, we haven't known – but new functional MRI (fMRI) methods have helped increase our understanding of the neural processes behind diagnostic decision-making (3,4). We spoke to Marcio Melo, lead author of the study, to find out more…

### Anatomy of a diagnosis

Schematically, the traditional view of the diagnostic process is:

1. Diagnostic cues evoke diagnostic hypotheses.
2. The investigative process (including history taking, physical examination, and diagnostic testing) confirms or disproves each hypothesis in the differential diagnosis through an interactive process, until a final conclusio is reached.
3. Based on that conclusion and any additional relevant information, a treatment is chosen.

But the decision-making process is not as simple as it looks, according to Melo. There are actually many, many choices made in the course of a single medical assessment. After the patient presents with the initial complaint, each step in the diagnostic investigation demands a decision: what question should I ask next? What exam should I order? With the degree of uncertainty we can expect from a diagnostic assessment, especially in its early stages, the physician needs to decide at each point in the process which of the many possible paths to choose.

Even when we factor in all of these branching points, we haven't fully encompassed the complexity of the process. It's certainly not as linear in real life as it looks on paper. For instance, the more complicated the disease, the more likely it is that a doctor will have to take a few steps back along the diagnostic pathway to find the right route. And, of course, we often find ourselves jumping ahead as well as back – the study's results indicate that treatment alternatives are

often evoked during the diagnostic assessment, before a final diagnosis has even been reached. It's clear that the real process does not follow the standard description.

But as powerful as fMRI is, that level of complexity would have made it difficult to observe what was actually going on in the brain during the process of making a diagnosis. So, to test their hypotheses using fMRI, Melo and his colleagues needed participants to follow a simplified diagnostic process based on only key information – allowing them to investigate the brain mechanisms underpinning that process. But Melo believes it is worth keeping in mind that, even in everyday medical practice, things are much more complicated than they appear here…

## What did we see?

First, that the way the brain identifies and names objects in everyday life is very similar to the way it tackles a tough diagnosis (see Figure 1). Second, that the more conclusive information the brain has at the start of the diagnostic process, the less attention it's likely to pay to the rest of the process, meaning that vital information may be overlooked – an important cause of diagnostic errors. And third, that there's a distinct shift in brain activity between reaching a conclusion and speaking it aloud (see Figure 2) – suggesting that we may, in fact, become more aware of our own conclusions simply by verbalizing them.

## Naming disease

Why does a complex process like disease diagnosis resemble something so seemingly simple as naming objects? Identifying and naming things in everyday life is, in fact, a highly complex neurocognitive process; its triviality in our lives is what gives it the illusory appearance of simplicity. In domains where identification and naming demand

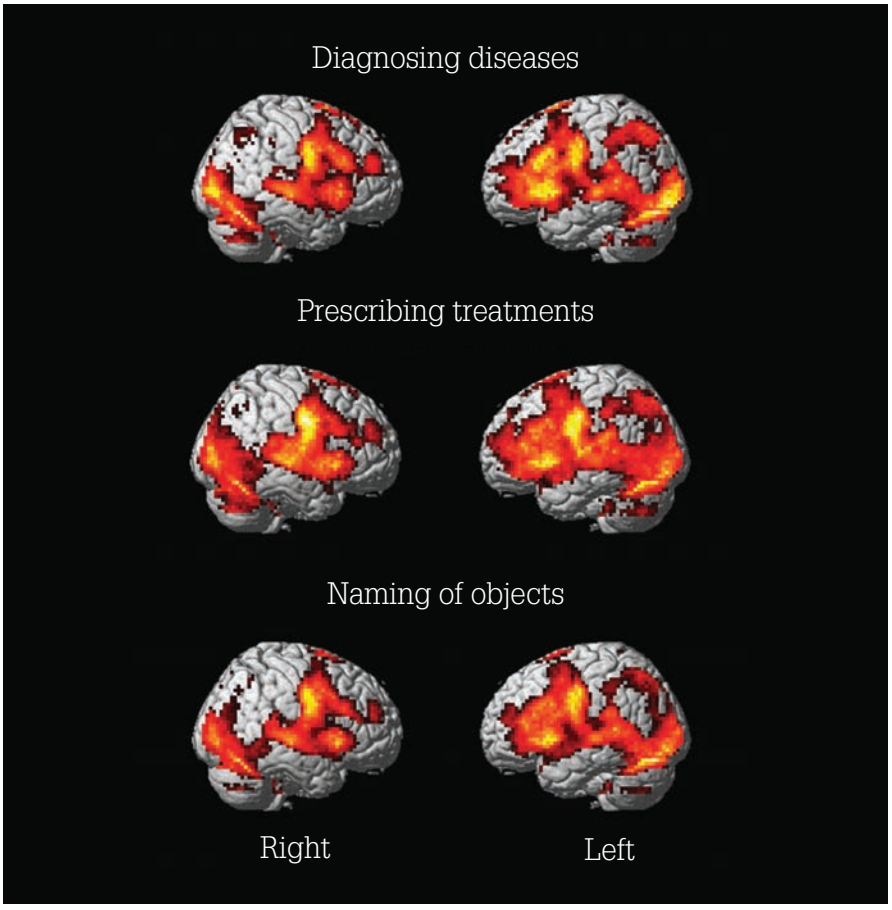


Figure 1. A comparison of brain function, as seen via fMRI, when diagnosing disease (top), prescribing treatment (middle), and naming everyday objects (bottom).

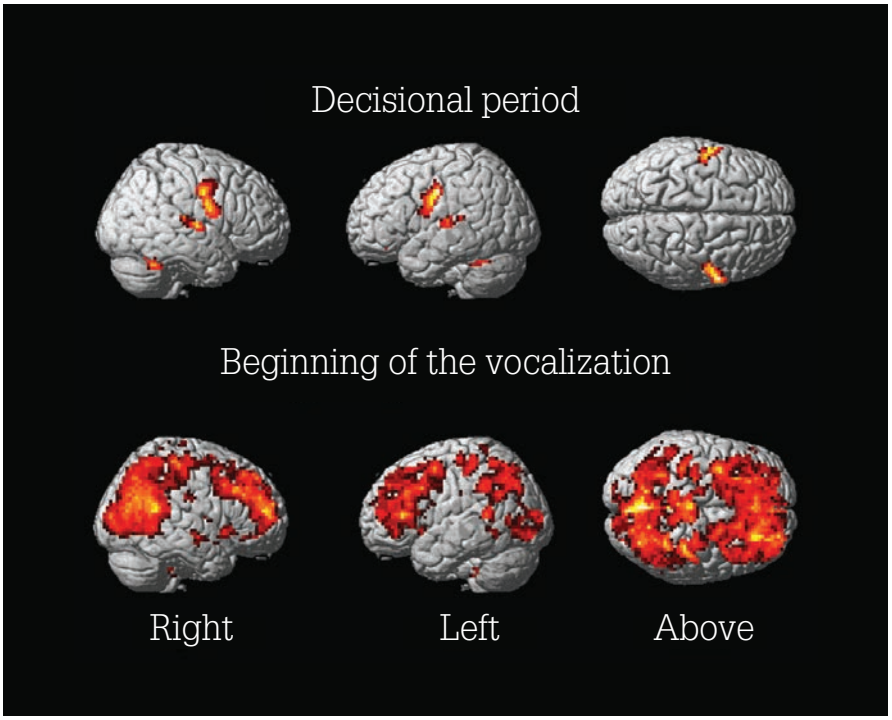


Figure 2. The change in brain activity between the decision-making period (top) and speaking the conclusion aloud (bottom).

a high level of expertise – like, for instance, botany – it becomes clearer that it poses quite a challenge for the brain. Conversely, disease diagnosis doesn't always seem complicated, either; many illnesses are easily identified by laypeople who have familiarity with them. After a child has had a few cases of tonsillitis, most parents have already guessed the problem by the time they've made a doctor's appointment.

And so, the initial hypothesis was that diagnosing diseases was similar to identification and naming in everyday life. Other people had already proposed that diagnosis is a categorization process, but the team's study on radiological diagnosis was the first experimental demonstration that the brain systems involved in diagnosing and naming were similar. Radiology seemed to them the obvious place to start because, at the time, the neural basis of naming things using visual stimuli had already been extensively investigated with fMRI.

## Talk it out

While observing the diagnostic process from start to finish, Melo's team encountered one completely unexpected finding – a large-scale switch of brain activity between the end of the decisional period and the beginning of the vocalization of the responses. It turns out that mentally making the diagnosis involves quite a different pattern of brain activity to actually speaking the diagnosis out loud. The activity shift takes place in several brain structures related to both awareness and auditory monitoring; careful analyses led them to postulate that this is the neural correlate of the doctors' becoming aware of the diagnoses they were making. If that conclusion is correct, then it's something that inevitably occurs every time a physician makes a diagnosis, right or wrong. From a broader perspective, this is how we become aware of our own thoughts. It's a counterintuitive conclusion – we've been taught to expect that we first think, and only then speak our thoughts out loud, but it seems that perhaps it's the speaking that actually materializes the thought.

It has been proposed that being reflective (for instance, by talking to oneself) could help decrease diagnostic error by increasing physicians' awareness of their thought processes and conclusions. The supporting evidence for this approach is inconclusive, though, so more research is needed to determine whether or not the benefits are worth the time investment. After all, it's difficult to pause and reflect when your consultations have an average duration of 10–15 minutes, as is common in primary care.

## A better way to diagnose

So how can all of this knowledge improve diagnostic accuracy? Or, put another way, what should pathologists and other diagnostic professionals learn from Melo's work? The researchers observed that highly specific information at the beginning of the diagnostic process – reducing uncertainty about the final diagnosis – decreases activity in brain regions involved in attention. This reduction of attentional monitoring may cause doctors to end their diagnostic investigations too soon, and could even lead to missing significant abnormalities. A similar process, called "satisfaction of search," takes place in the visual domain when a diagnostician finds one prominent abnormality, which increases the probability of missing other lesions. But how can we overcome such biases? Melo's suggestion is to present a list of differential diagnoses at the beginning of the diagnostic assessment instead of later in the process – thereby increasing uncertainty regarding the final conclusion and potentially preventing premature diagnostic closure. This can be implemented by employing user-friendly computerized diagnostic support systems if you have access to them. "I firmly believe," says Melo, "that tools like these can help guide the diagnostic process while reducing errors and biases, and will become increasingly helpful in improving medical accuracy."

Until now, the team's studies have focused on situations in which the diagnosis is straightforward. Their next step will be to investigate the brain mechanisms involved in conditions that require more complex diagnostic processes. The investigation and understanding of the neural processes physicians and healthcare professionals employ in making diagnoses and prescribing treatments are in their earliest stages. "I'd like to see them examined in much more detail," says Melo, "but we'll need more resources devoted to these kinds of studies. I think that, as our understanding of disease advances and our brain visualization technologies improve, studies like ours will become increasingly important in helping us better comprehend the inner workings of the diagnostic process – and mitigate medical error."

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# Cracking Cancer's Code

**Sitting Down With... George Calin, Professor**, Department of Experimental Therapeutics, and Co-Director, The Center for RNA Interference and Non-coding RNAs, Division of Cancer Medicine, The University of Texas MD Anderson Cancer Center, USA.

How did you get into studying the role of RNA in cancer?

It was entirely down to chance. I was born in Romania, which at that time was a Communist country. The study of genetics was frowned upon, but I was lucky to have the geneticist Dragos Stefanescu as a mentor; he taught me cytogenetics, but also how to do good science. I wanted to study abroad, so I wrote 120 letters to 120 scientists all over the world – and ended up moving to Italy, where I studied molecular genetics in Massimo Negrini's laboratory before moving to the United States. I was at Kimmel Cancer Center in Philadelphia in Carlo Croce's laboratory when we discovered a link between microRNAs and human cancer. It was the first time a link had been made between any type of non-coding RNA (ncRNA) and a human disease.

But the initial paper was rejected, right?

Yes, by a famous journal in just 12 hours. Attached was a simple note saying that our discovery was random, and because it wasn't proven that microRNAs were important in cancer, our work would be better suited to a smaller journal. Today, that paper has probably been cited more than 4,000 times, and there are over 30,000 papers in PubMed on microRNAs or long ncRNAs and cancer. That discovery opened the door, both for me and for many thousands of other scientists to enter an exciting and entirely new field of molecular oncology. But much of it was actually luck and perseverance!

In 1992, when I was a young student in Romania, the first scientist who helped me write a research essay was Thomas Cech, who received a Nobel Prize for his work with RNA and his discovery of ribozymes. It's funny to think that now, 25 years later, I am working in the field that Thomas popularized: the role of RNAs in biology, and in pathology in general.

You aim to produce the first complete catalog of human ncRNAs. How close are you?

Day by day, I think I am getting further away! The genome is complex, and there have been many surprises. A colleague at Thomas Jefferson, Isidore Rigoutsos, and I recently had a paper accepted on the topic of human- and primate-specific ncRNAs that have no homology with other species. Essentially, these transcripts represent a fingerprint of humanity, which could have big implications for research – many scientists, including me, spend a lot of time and money using mouse models of human cancers. But as geneticists and clinician-scientists, we know that mouse models don't reproduce human cancers very well. Without a deep and meticulous understanding of the genome and how it functions, I don't believe we will make big advances in developing new biomarkers and therapeutics for cancer. I hope our new ncRNA research will help to bridge that gap. We are heading in the right direction, but it's going to take a long time. Luckily, the journey is fascinating and full of unexpected turns, which is great – it keeps life interesting!

Why focus on cancer in particular?

If you or I were to go to a doctor, receive a cancer diagnosis, and be told we might only have weeks or months left to live, it would be absolutely devastating news. Patients with advanced cancer are in serious need of a better understanding of their disease, which can lead to better management and potentially to new treatments. And that's why I choose to focus on identifying the mechanisms of metastasis and the ways we can block the spread of cancer cells.

Another reason to focus on cancer is the potential to use ncRNAs to predict cancer occurrence. It's my hope that, one day, we'll be able to take a body fluid, such as plasma, and develop a relatively noninvasive test that looks at ncRNAs and tries to identify which people will get lung cancer, prostate cancer, and so on. Of course, these are goals that many researchers have been pursuing – some looking at DNA, others at proteins or RNA. I believe that combining these biomarkers will yield the biggest advantage. We can't fully treat any type of disease if we don't understand it, and nucleic acids – both coding and non-coding – are like the different words of a sentence. We have to understand it all and look at it in the right way to truly understand a disease.

*"Nucleic acids – both coding and non-coding – are like the different words of a sentence."*

When you look back at your career, would you do anything differently?

I don't think there's anything I would change. My path has led me to one of the biggest cancer centers in the world, where I'm lucky to have great collaborators and colleagues. But so much of my success is down to other people that I cannot thank them enough. It's important to thank my mentors, because without them, I wouldn't be where I am. We should all thank our fellows more as well – no matter how clever you are, how good your ideas are, or how much funding you bring, you are in debt to the people in your lab who work to make your ideas a reality. So, I'd like to thank both those who came before me and those who came after for making my work possible.

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