Testing for platelet function using platelet-rich plasma

Amy Carpenter Aquino

July 2021—Identifying severe disorders of primary hemostasis is relatively straightforward for most coagulation laboratories, but the more prevalent disorders with less severe bleeding and less overt diagnostic abnormalities are trickier, and platelet function testing using platelet-rich plasma remains the gold standard.

Geoffrey Wool, MD, PhD, in an AACC virtual session last year, presented some of his laboratory's cases to illustrate the use of light transmission aggregometry and a modification called lumi-aggregation.

"The principle of LTA is based on the idea that light passes more easily through a clear than a turbid solution. It's pretty basic," said Dr. Wool, medical director of the coagulation laboratory and associate professor of pathology, University of Chicago.

He compares this principle to a plateletpheresis unit seen in the blood bank, which is turbid and through which light can't pass easily. But a rapidly centrifuged plasma sample is clearer. One is platelet-poor plasma (PPP); the other is platelet-rich plasma (PRP), "and the difference in the light passage is the underpinning of light aggregation testing."

Dr. Wool

"We're going to take aliquots of that PRP, add various platelet activating agents, and then measure how well or not the platelets respond," he said. Platelet activation is measured by clumping (or aggregation). As platelets are activated, their surface receptors for fibrinogen (GpIIb/IIIa) adopt an active conformation and platelets form aggregates with fibrinogen. As the platelet aggregates form, light passage through the cuvette of PRP increases.

Additionally, when platelets are activated, their granules will be brought to the membrane surface to fuse and degranulate. Release of ATP is often used as a marker for dense granule release.

"What we're taking advantage of in lumi-aggregation," Dr. Wool said, "is measuring the ATP from the platelets by adding firefly luciferin and luciferase and then measuring the visible light that's generated when the ATP is released from the dense granules."

Light transmission aggregometry (LTA) is not a perfect test, he said. "It's performed under nonphysiologic conditions in a stirred solution under relatively low shear conditions. In contrast, we know that platelets function most potently in vivo to bind to surfaces under high shear stress, such as a damaged surface of an artery or arteriole. That's where they're doing a lot of their work, so with that comparison we can see how relatively nonphysiologic LTA testing is."

Dr. Wool and his colleagues at the University of Chicago use lumi-LTA, and one of their cases was that of a Puerto Rican woman worked up for lung transplant. She had lifelong easy bruising and prolonged bleeding with minor injuries or dental or surgical procedures. She had no children but had a brother with increased bleeding. Her platelet count, mean platelet volume, and other CBC results were normal.

Her platelet aggregation results showed mildly to moderately reduced aggregation responses to a variety of agonists, including epinephrine, arachidonic acid, thromboxane analogue, thrombin receptor activating peptide,

and collagen. Her adenosine diphosphate (ADP) and ristocetin aggregation responses were normal. In marked contrast, “her ATP release was uniformly absent,” Dr. Wool said, implying a severe absence or dysfunction of her delta granules.

“So her diagnosis was a dense granular deficiency called Hermansky-Pudlak, and she has type 1,” which is more common in people of Puerto Rican ancestry, he said. “Type 1 is associated with pulmonary fibrosis as well,” explaining her need for lung transplant.

Hermansky-Pudlak is an autosomal recessive disorder characterized by dense granule deficiency and a mutation in the *HPS* gene, and, in addition to bleeding, it is associated with oculocutaneous albinism. “The ADP and ATP in the dense granules aren’t coming out to stimulate the other platelets in the PRP. So the aggregation responses are reduced but variably, depending on the strength of the agonist you’re using. But the ATP release will be uniformly terrible,” Dr. Wool said.

In another case, a 39-year-old woman who had childhood epistaxis, lifelong bruising predisposition, and menorrhagia had a normal platelet count and MPV. Her red and white blood cell parameters were unremarkable, and her von Willebrand disease panel was negative.

Her platelet aggregation responses were reduced to most agonists, most severely for arachidonic acid. Secretion was significantly reduced for most agonists. The strong activators—thromboxane analogue, thrombin receptor activating peptide, and the calcium ionophore A23187—revealed normal to mildly reduced responses with appropriate secretion, he said.

“If we put all of her responses together, the worst response was the arachidonic acid. The next worst were the epinephrine responses, then collagen at two micrograms per mL,” Dr. Wool said. “This is a classic pattern of absent response to arachidonic acid, poor response to epinephrine, and poor response to low concentration of collagen, but relatively normal response to a high concentration of collagen that should make you think of aspirin.” Aspirin (acetylsalicylic acid) is a cyclooxygenase inhibitor that leads to total unresponsiveness to arachidonic acid, he said, and also leads to poor responsiveness to weak platelet agonists.

The juxtaposition of a “quite terrible” response to arachidonic acid and a normal response to thromboxane analogue U44619 should “hammer home the thought that this might be aspirin related,” he added.

The cyclooxygenase metabolic pathway uses a polyunsaturated fatty acid substrate such as arachidonic acid and eventually produces the prostaglandin thromboxane A2. If normal platelets are exposed to exogenous arachidonic acid, he explained, their cyclooxygenase and thromboxane synthase enzymes will produce thromboxane and lead to platelet aggregation. In aspirinated platelets, on the other hand, “if we feed in arachidonic acid at the top of the cyclooxygenase pathway, nothing happens.” If synthesized thromboxane (or analogue) is provided, however, aspirinated platelets can still respond well.

“So the pattern of platelet responses for this patient is telling you there’s a defect in the pathway between arachidonic acid and thromboxane,” Dr. Wool said. “This should make you think the patient was taking something with aspirin until proven otherwise.”

Findings were persistent on a second lumi-aggregation study. In extensive discussions with the patient, she denied using aspirin or over-the-counter products that can contain aspirin. “We even did a salicylate screen in the chemistry lab, and she had a negative screen.”

“So this patient has what’s called an aspirin-like defect,” Dr. Wool said (Rao GHR, et al. *Am J Hematol.* 1981;11[4]:355–366). “She had a congenital platelet disorder of either the cyclooxygenase pathway or the thromboxane synthetase pathway that prevented her platelets from responding to arachidonic acid or making thromboxane. Once you added thromboxane or an analogue of it, the platelets responded perfectly well. But they weren’t able to make their own thromboxane.”

The case of a 56-year-old woman with excessive bleeding after a cervical electrical excision procedure illustrates

the importance of patient preparation for LTA. The patient had no prior history of bleeding before the procedure and she had normal CBC results. On her current medication list were levothyroxine, liothyronine, and lansoprazole, as well as several dietary supplements, each containing many herbal ingredients.

Dr. Wool described the patient's platelet aggregation responses as generally normal, but with a significantly deficient response to thromboxane analogue U46619: Aggregation reached only to 15 to 30 percent and then platelets completely deaggregated. This shows that the platelet clumps were poorly/weakly made and fell apart under the stirring conditions of the LTA. Responses to other agonists were generally normal.

"She seemed to have an isolated deficiency of response to thromboxane, which is not a common pattern," Dr. Wool said. "Even more bizarrely, if she had a significantly reduced response to thromboxane, you would think the response to arachidonic acid would be correspondingly reduced, but that's not what we saw." The patient's arachidonic acid response was only mildly reduced and not nearly as much as her response to thromboxane. "It was a strange aggregation pattern with absent response to thromboxane."

The peripheral blood stain was even odder, he said. The majority of platelets lacked the blue granular staining indicating alpha granules in a Wright-Giemsa–stained blood smear. "There were nicely granular neutrophils, relatively normal red cells, and there was a variety of platelet morphologies. Some of them looked relatively normal with blue granules in them, but most of them were pale, consistent with significant reduction in the alpha granular content."

Dr. Wool said his team was surprised to see such a severe platelet function defect, called "gray platelet syndrome." "And the thromboxane response as a standalone abnormality was rather odd. So we asked to do this study again once the patient was not taking her dietary supplements."

When the patient had been off of supplements for three weeks, a repeat LTA showed "a completely normal response to all the agonists, including thromboxane analogue," he said. Platelet morphology was also normal. "We even did reflex flow cytometry" because of the suspected gray platelet disorder. Results showed that the P-selectin exposure, a marker of alpha granule release, was normal after ceasing supplement use.

The diagnosis was an acquired platelet defect associated with dietary supplements. "Certainly an interesting situation," Dr. Wool said, and it suggests that the abnormal bleeding after her medical procedure could have been due to her dietary supplements.

One of the drawbacks of platelet aggregation testing is that it requires relatively large-volume phlebotomy (20–40 mL), and the sample must be freshly collected, hand-delivered, and processed rapidly, Dr. Wool said. It requires validated reference intervals, derived from local healthy control donors.

Blood samples should be drawn with minimal or no venostasis, using a 21-gauge or, ideally, larger needle into 3.2 percent buffered citrate polypropylene or siliconized glass tubes, filled to appropriate 9:1 ratio, gently mixed. A 3.8 percent sodium citrate concentration is also acceptable, as long as use is consistent. "If you develop a donor reference range using polypropylene, you need to keep using polypropylene. If you develop a donor range using 3.2 percent sodium citrate, you need to keep using 3.2 percent sodium citrate. Make sure your hospital or health care system doesn't have a variety of tube types in different locations."

Blood processing must be immediate to prevent platelet activation. LTA maximum aggregation is significantly reduced in samples in which platelet aggregate formation was induced prior to analysis (Hechler B, et al. *Res Pract Thromb Haemost.* 2019;3[4]:615–625; Sugawara E, et al. *J Stroke Cerebrovasc Dis.* 2019;28[4]:1001–1006).

The platelet-rich plasma is prepared by gentle centrifugation at room temperature for 10 to 15 minutes at 70 to 200 g with no brake. The PRP supernatant is removed and placed into a stoppered plastic/siliconized glass tube, and further centrifugation of the remaining blood at 2,700 g for 15 minutes produces the platelet-poor plasma (Alessi M-C, et al. *J Clin Med.* 2020;9[3]:763).

"The centrifugation needs to be done with some thought and care" because higher centrifugation speeds are

associated with decreasing PRP platelet count ($P<0.001$) and mean platelet volume ($P<0.001$). High spin results in loss of larger platelets, which tend to be the freshest and youngest and quite responsive platelets, Dr. Wool said. "If you lose those, you can certainly see a decline in aggregation response" (Merolla M, et al. *Int J Lab Hematol.* 2012;34[1]:81–85).

The PRP platelet count should be above 150×10^9/L; below that threshold, responses below normal could be due to low platelet count alone, though normal studies are still informative.

"There is a split in the field about whether we should try to adjust the PRP platelet count," Dr. Wool said, meaning dilute higher platelet counts down to a standard PRP count (such as 250×10^9/L). Standardizing PRP platelet counts normalizes the frequency with which platelets bump into each other in the cuvette, and therefore tightens the spread of the normal range of platelet aggregation responses. While some laboratories perform platelet adjustments by diluting PRP with platelet-poor plasma, "at the University of Chicago, we do not." This decision was based on studies showing that the decrease in platelet aggregation observed after dilution of PRP with platelet-poor plasma is not due solely to the decrease in platelet count but to the inhibitory effects on platelet aggregation of substances contained in the PPP (Cattaneo M, et al. *Haematologica.* 2007;92[5]:694–697).

The final case Dr. Wool presented was that of a 13-year-old male with a history of easy bruising and variable thrombocytopenia (from 59 to 121×10^9/L) throughout his time at the University of Chicago Medicine. The patient required suturing and packing after a dental extraction, and male relatives on his mother's side had had bleeding problems. A prior platelet lumi-aggregation study at the University of Chicago about six years previously found a significant dense granule secretion defect.

Current CBC results showed thrombocytopenia (platelet count, 86). His MPV was normal, but he had a slight anemia (RBC, 5.03×10^{12}/L; hemoglobin, 12.1 g/dL), which was slightly microcytic (MCV, 80.9 fL) for the reference range.

On the peripheral blood smears, "his red cells showed a bit of polychromasia" and platelets that Dr. Wool described as "striking": some large, most of them too pale, lacking their alpha granules.

Aggregation responses were generally mildly reduced, with low concentration collagen being more significantly abnormal. In contrast, ATP secretion response was uniformly absent.

"Our diagnosis was a severe dense granule secretion defect, which we had seen before, and then morphologically abnormal platelet alpha granules, which had not been well described previously," he said. This implied a deficiency or abnormality of both types of platelet granules. "So we wanted to send this for electron microscopy," which is often paired with lumi-LTA. "Even with the gold standard assay, you still are not able to reach every diagnosis using the LTA technique alone."

The EM findings included markedly decreased platelet dense granules (0.3 dense granules/platelet; reference range ≥1.2/plt); also seen were decreased numbers of alpha granules and atypical large or fused alpha granules.

Research flow cytometry confirmed the significant reduction of alpha granule content. "The patient's ability to fuse and release his platelet alpha granules was severely impaired compared to a normal donor," Dr. Wool said.

An additional ATP secretion response test, developed by University of Chicago resident Joseph H. Cho, MD, PhD, was performed (Cho JH, et al. *Am J Clin Pathol.* 2021;155[6]:863–872). It uses less than 1 mL of diluted whole blood and improves the ability to detect low concentration ATP secretion over the standard reagent luciferin-luciferase assay for ATP release, which can't measure ATP release precisely below 50 pmol.

"The optimization that Dr. Cho achieved in our laboratory has allowed us to measure down to five picomoles of ATP before achieving that degree of imprecision seen with the standard reagent set," Dr. Wool said. "It's quite an optimized assay." And with the new assay, "we can see the patient's platelets put out a little bit of ATP in response

to TRAP [thrombin receptor activating peptide, 40 µM] and collagen [5 µg/mL],” he said. “This confirms the presence of ATP-containing dense granules, but in significantly reduced number.”

Molecular analysis revealed the patient had a *GATA1* mutation in exon 4. “It’s in a highly conserved amino acid position,” and the p.Arg216Gln change impairs recruitment of the *TAL1* complex, impacting transcriptional activation by *GATA1.* This sequence change has been reported in several families with X-linked thrombocytopenia with thalassemia, Dr. Wool said. “Our final diagnosis was an alpha/delta storage pool disorder (αδ-SPD) as part of an X-linked thrombocytopenia with thalassemia syndrome,” though the patient only had mild evidence of thalassemia.

“That was quite an interesting case,” he said. “We had to put a lot of different research and ancillary techniques together to get to that diagnosis.”

Of the predictive value of LTA, Dr. Wool said that an abnormal LTA “does allow you to pretty significantly rule in a platelet function defect,” so long as drug interference is ruled out (as discussed).

The ability of a normal lumi-aggregation result to rule out platelet dysfunction, while not 100 percent, “is certainly better than other platelet function tests on the market,” Dr. Wool said. In a study from Birmingham, England, 111 unrelated participants with suspected platelet function defects had intensive testing, including LTA. Despite this impressive battery of testing, nearly 40 percent could not have a platelet function defect identified. Three research assays did not add any diagnostic ability in subjects with a normal lumi-aggregation study (Dawood BB, et al. *Blood.* 2012;120[25]:5041–5049).

The pattern seen with responses to different agonists can be helpful in narrowing the dysfunctional pathway or receptor, and the presence of reduced response to more than one agonist is a more powerful predictor of a real defect than is reduced responsiveness to just one agonist (Hayward CPM, et al. *Int J Lab Hematol.* 2019;41[suppl 1]:26–32).

“Just be aware,” he said, “that epinephrine and low concentration ADP are the weaker agonists, and even normal people can have some reduced response to them.” In their 2019 study, Mezzano and his coauthor analyzed platelet aggregation and plasma von Willebrand factor in healthy controls and in patients with platelet function defects, type 1 von Willebrand disease, and bleeding of undefined cause. They found “impaired LTA with 10 µM epinephrine and reversible aggregation with 4 µM ADP in 13.7% of 299 controls,” they wrote, “and, accordingly, considered this combination of defects insufficient for the diagnosis of genuine PFD” (Mezzano D, et al. *J Thromb Haemost.* 2019;17[2]:257–270).

Dr. Wool advises interpreting LTA results “with some knowledge and skill, knowing what the caveats and pitfalls are.”

Amy Carpenter Aquino is CAP TODAY senior editor.