Immunol Res (2009) 44:42–53
DOI 10.1007/s12026-008-8069-2

The thrombocytopenia of WAS: a familial form of ITP?

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Published online: 15 October 2008

Abstract In the first report of the concurrent immunodeficiency, thrombocytopenia, and eczema that we now call the Wiskott-Aldrich Syndrome (WAS), Alfred Wiskott asked whether it could be a familial form of Werlhof's disease (now called ITP). This review summarizes what is known about platelet production, consumption, and function in clinical and murine WAS. Both platelet production and consumption are affected by WASP deficiency. Likely molecular mechanisms have been identified for the former process, but remain problematic for the latter. Recent data in a murine model suggest that WASP deficiency could increase both the incidence of antiplatelet antibodies and susceptibility to their enhancement of platelet consumption. Wiskott's original speculation about the relationship between WAS and ITP may need to be reconsidered.

Keywords Platelets · Wiskott-Aldrich · ITP

The clinical syndrome we call the Wiskott–Aldrich Syndrome (WAS) was first described in 1936 in a report titled "Familiärrer, angeborener Morbus Werlhofii?" [1]. In it, Alfred Wiskott described three brothers with the clinical findings now felt to be textbook elements of the syndrome: immunodeficiency, thrombocytopenia with unusually small platelets, and eczema. It was perhaps understandable that Aldrich and his colleagues were not familiar with the German clinical literature when they described, 18 years later, similar findings in one child and inferred an X-linked inheritance from an extensive family history [2]. What should not have been lost is Wiskott's original question: was this a congenital form of Werlhof's disease, what we now call immune (or, in some usages, idiopathic) thrombocytopenic purpura (ITP)?

Both Wiskott and Aldrich would be proud how far our understanding of their syndrome has come. The gene was identified in 1994, and mutations in hundreds of WAS patients

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have been sequenced and correlated with variations in their clinical presentation [3]. Biochemical functions of the WAS protein (WASP) have been characterized in detail, and roles played by WASP in a number of signal transduction pathways, and in several cell types, have been demonstrated (reviewed recently by Takenawa and Suetsugu [4]). These studies have spawned related work in a family of WASP-related proteins that go well beyond the realm of hematology. WASP(−) mice have been derived by two labs [5, 6], show some but not all of the features of clinical WAS, and have been used to demonstrate the feasibility of gene therapy for WAS [7–9].

Werlhof, by contrast, might not be as thrilled. After ITP was shown to be largely autoimmune in nature in 1951 [10], all that remained was the development of a sensitive and specific immunologic assay in order to make it a rigorously defined clinical entity. That assay apparently has not yet arrived, and ITP remains a clinical 'garbage can' category arrived at by ruling out other etiologies, not all of which are specified [11].

There are at least four reference frames in which the thrombocytopenia of WAS might be understood. The first, and most employed up to now, is that of platelet kinetics: Are there abnormalities in what we know about platelet consumption and platelet production which are induced by WASP deficiency? The second is that of platelet function: Are there abnormalities in platelet aggregation, signal transduction, or metabolism? The third, and potentially the most satisfying is the framework of WASP function: Can signal transduction pathways known to involve WASP in other contexts be shown to be involved in generating the thrombocytopenia and small platelet size characteristic of clinical WAS? And a fourth framework, as Wiskott suggested, is ITP: Are there features of the thrombocytopenia of WAS that parallel those seen in ITP, and might equating these two poorly understood syndromes somehow clarify the mechanism responsible for one or both?

Platelet kinetics in WAS: consumption rates and processes

The question of whether WASP deficiency primarily affects platelet production or destruction rate has been addressed in several clinical studies. Allogeneic platelets are consumed at a normal rate in WAS patients, an observation interpreted at the time to mean that the thrombocytopenia was due to impaired platelet production [12]. Grottum et al. [13]. found that the consumption rate of autologous [51] Cr-WASP(−) platelets in two WAS patients with moderate thrombocytopenia (platelet counts >50,000/μl) was extremely rapid, with over 90% of platelets disappearing from circulation within 48 h. By contrast, WASP(+) platelets infused into one of these patients had a near normal consumption rate, with approximately 90% gone at 7 days. Baldini [14] took the process a step further and demonstrated rapid consumption of two WASP(−) platelet preparations in normal volunteers—again showing over 90% removal from circulation within 2 days. Ochs et al. [15], however, found only a modest increase in autologous platelet consumption rate in four WAS patients (three of whom had platelet counts which were predominantly below 50,000/μl). This group concluded that both platelet production and platelet consumption were affected by WASP deficiency. Rapid consumption of allogeneic platelets was also seen in two severely thrombocytopenic cases, with rates comparable to that of autologous platelets. It required subsequent studies of normal platelet kinetics to make sense of the latter observation: consumption rate increases in patients with severe thrombocytopenia even if the latter is due primarily to reduced production [16].

In the murine model, WASP deficient platelets are consumed more rapidly than WT platelets in WT recipients, suggesting again an intrinsic platelet defect [17]. Both WT and

WASP(−) platelets are consumed more rapidly in WASP(−) than WT recipients, which might be expected given the former's significant splenomegaly. Splenectomy improves platelet counts in almost any thrombocytopenia, and this is the case in both clinical and murine WAS. In the former case, there is subsequently a high incidence of ITP (23% in one study [18], see below). In the murine model, splenectomy improves platelet count but does not render it equivalent to that of splenectomized WT mice, and it does not normalize the consumption rate of WASP(−) platelets [17]. These findings indicate that whatever the root cause of rapid consumption of WASP(−) platelets, consumption or sequestration in the spleen is only partially responsible.

Platelet kinetics in WAS: production rates and processes

In contrast to studies of platelet consumption, platelet production is typically not measured directly. In addition to inferences based on platelet count and consumption rate, efforts have been made to characterize the fraction of immature platelets via thiazole orange binding assays ("reticulated platelets," or RP); megakaryocyte number has been estimated; and thrombopoiesis has been studied directly both ex vivo and in vivo.

Reticulated platelets are platelets which take up slightly more thiazole orange (TO) than others, although the TO positive and negative populations overlap significantly. In vivo kinetic studies have shown that RP are produced and consumed more rapidly than mature platelets [19], and this provides a qualitative guide for placement of gates between the TO^+ and TO^- populations. Quantitatively the issue is more difficult, which may explain the over tenfold variation in normal ranges of percent RP in the literature [20, 21]. Kinetic data suggest that mature platelets are initially produced as RP which then mature into the non-reticulated form, a process analogous to immature red cells (reticulocytes). RP numbers and turnover rate, however, are insufficient to account for the turnover of mature platelets [17, 22], indicating that at least some, and possibly most, platelet production does not involve RP. The %RP was reported to be reduced in WAS patients in one series [23] and increased in another [24]. Their absolute numbers are reduced in WASP(−) mice, although the %RP is increased in a subset of these mice [17] (a finding which suggests the presence of anti-platelet antibodies, see below). Given the above caveats and the disagreement between different reports, it is hard to know how to interpret these findings.

Megakaryocyte numbers have been reported to be normal in bone marrows of most [13, 25] but not all [15] WAS patients. Splenic EMH has been reported in only a few cases (2 of 12 in one series [26]). Although splenic EMH was found more frequently in another report, megakaryocytes were noted to be relatively under-represented [27]. These are unexpected results in the context of chronic thrombocytopenia, and in conjunction with a report of impaired ex vivo megakaryopoiesis [28] they suggest impaired megakaryopoiesis in most WAS patients. WASP(−) mice, in contrast, show an increased number of bone marrow megakaryocytes [17, 29], and ploidy studies show no abnormalities in megakaryopoiesis [17]. Splenic EMH is a normal finding in mice, and in WASP(−) mice it is markedly increased in association with significant splenomegaly [30]. Taken together, these results suggest that impaired megakaryopoiesis in WAS patients could contribute significantly to the thrombocytopenia, and that the ability of WASP(−) mice to increase megakaryopoiesis could contribute to the reduced severity of the thrombocytopenia seen in that model.

Studies of thrombopoiesis in WAS have been limited by our limited understanding of the process itself. It was only in 2007 that the remarkable in vivo imaging studies of Junt et al. [31] showed that murine platelets are produced by intrasinusoidal extension and

fragmentation of megakaryocyte processes ("proplatelets"). This work supports the in vivo relevance of previous studies of proplatelet formation by WASP deficient megakaryocytes. Although one of these concluded that their formation is normal ex vivo [32], others have reported that it is morphologically abnormal ex vivo [28, 33], inhibited when WASP activity is blocked [34], and active but premature in vivo [29]. In the latter study, dysfunction of megakaryocyte chemotaxis in response to SDF-1 was seen, as well as defective inhibition of proplatelet formation due to dysfunction of a specific signaling pathway between type I collagen and the megakaryocyte integrin $\alpha 2\beta 1$.

Platelet function in WAS

It is difficult to find solid clinical grounds on which to base studies of altered platelet function in WAS. If a *reduced* aggregation response contributed to the incidence of hemorrhage in WAS patients, one would expect that splenectomy, which significantly increases platelet counts in WAS, would have at best a partial impact on the incidence of hemorrhage. That is not the case; in a series of over 60 WAS patients who underwent splenectomy, significant subsequent hemorrhages were only seen in patients who developed ITP [18]. The latter, as noted previously, has a rather high incidence in this context. If an *increased* aggregation response contributed to the thrombocytopenia of WAS, one might expect clinical or pathologic findings consistent with increased thrombosis to occur. None have been reported.

Nonetheless, multiple aggregation studies of WASP(−) platelets in response to several agonists have been published. These include reports of a reduced response to multiple agonists [35], a normal response to multiple agonists [36], a mildly increased response [37], and an increased calcium influx which would be expected to cause an increased response [38]. These studies are both difficult to perform (because those patients with the most severe thrombocytopenia have the fewest available platelets for study) and difficult to interpret, as it is unclear how to distinguish between a primary effect of WASP deficiency and a secondary one associated with the reduced size and age of the platelets.

Platelet-derived microparticles are increased in number in the plasma of WAS patients [38]. This raises the possibility that progressive fragmentation of WASP(−) platelets could result in the observed microparticles, reduced platelet size, and presumably reduced platelet lifespan.

WASP function in platelets and megakaryocytes

An alternative approach to understanding how WASP dysfunction might induce a thrombocytopenia would be to predict, from what is know about WASP function in other cell types, what types of structural or functional abnormalities might ensue. One problem here is that the potential effects are legion: any cellular structure or function that involves actin polymerization might reasonably be suspect, a line of thinking that can encompass cell shape change, motility, trafficking, and a large number of signal transduction mechanisms. Evidence that WASP-dependent [39] or N-WASP-dependent [40] actin polymerization can regulate transcription opens the door to an even broader range of predictions.

In megakaryocytes, evidence that WASP deficiency results in dysregulated proplatelet formation (and therefore may affect platelet production rate or the size and composition of

nascent platelets) was cited above. In platelets, however, three groups have reported no impairment of shape change or f-actin polymerization in response to several types of stimuli [37, 41, 42]. Reduced f-actin in unstimulated WASP(−) platelets was reported by one group [23], although this could have been proportional to the reduced size of WASP(−) platelets. These results suggest that if WASP deficiency affects actin polymerization in platelets, it does so on a smaller scale or in a different context than has been studied thus far. One could envision, for example, an effect of WASP on the distribution of platelet membrane components which might not be evident in the type of study reported to date.

Does WASP play a role in any of the well-characterized signal transduction mechanisms that result in platelet activation? At the biochemical level it appears to, but no convincing linkage of these studies to overall platelet function has been made. For example, platelet WASP has been shown to undergo tyrosine phosphorylation in response to collagen [43], and collagen related peptide (CRP) [37], but WASP deficiency had little impact on platelet aggregation in response to CRP [37]. Platelet WASP has been linked biochemically to a number of other activities in platelets, including that of calpain [38, 43] the adaptor protein CRKL [44], the platelet surface complex αIIb-β3 [45], and CIB (a protein associated with αIIb-β3) [46], but no functional implications of WASP's absence in platelets have been linked to these molecules.

ITP

ITP is an odd disease because it takes some effort to understand what the "I" stands for. In clinical terms, it clearly stands for "idiopathic," and as a diagnosis of exclusion it should be thought of as a category rather than a disease. What makes ITP odd is the fact that we have known for many years that most patients with this diagnosis (at least, as it was functionally defined in 1951) carry "a thrombocytopenic factor" (later shown to be one or more immunoglobulins) in their serum [10]. ITP is, therefore, felt to be at least predominantly immune mediated, and for that reason the "I" is said to stand for "immune" in many publications and textbooks.

There have been many reports of increased platelet consumption rates in ITP (see for example Stoll [47], and references therein), which also appears to support an immune-mediated pathogenesis. But the increased consumption rates seen in cases of thrombocytopenia due to bone marrow failure (as noted above) [16] suggest that in at least some ITP cases the high reported consumption rate is a secondary effect. One study concluded that such a secondary effect could not account for the increased consumption rate seen in ITP cases [48]. There are, however, a significant number of reported cases in which platelet turnover rate is only mildly impaired [49].

Unfortunately, both in vivo platelet consumption assays and the rather remarkable methods used by Harrington et al. (direct injection of sera into volunteers, including the lead author) are somewhat impractical from a diagnostic standpoint. Antibodies specific for a number of platelet membrane proteins have been described in ITP cases, and one might think that a suitable in vitro assay could be found that would define "immune" thrombocytopenic purpura. But although several have been tested, none have demonstrated a high enough combination of sensitivity and specificity to warrant their use as a definitive assay for what we now call ITP (reviewed by Gernsheimer [50]). This suggests either that detection of antiplatelet antibodies is harder than it might seem, or that this diagnosis of exclusion comprises more pathophysiologic entities than was suggested by Harrington's work.

Detection of antiplatelet antibodies does in fact present unique problems. Assays using platelet targets would be hampered for several reasons: platelets carry a significant normal level of surface antibodies [51], and they can spontaneously clear additional surface antibodies [52, 53]. Those features could explain the lack of utility of direct testing for platelet-associated antibodies. Antigen-specific assays have also failed to achieve a high enough sensitivity and specificity to be clinically useful (reviewed by McMillan [54]). Possible contributing factors here include antigenic polymorphisms in the antigens tested (a large number have been described [55]), and the likelihood that the antigens tested make up only a fraction of those which are or can be immunogenic.

Could the category of ITP include pathophysiologic entities other than those involving antibody-mediated platelet clearance? It would be unwise to think otherwise about any diagnosis of exclusion. More than one study has implicated either reduced platelet production or an impairment in the expected increase in platelet production in the pathogenesis of ITP [48, 49]. And there are reports of an effect of antibodies seen in ITP on megakaryocyte function [56, 57]. An increased fraction of reticulated platelets has been described in ITP [21, 58, 59], but the caveats noted above for this method make these results hard to interpret. Myelodysplastic syndromes, such as the 5q(−) syndrome, can present primarily with thrombocytopenia, and one might think that such cases could be cleanly separated from the bulk of ITP cases on the basis of bone marrow morphologies and cytogenetics. But morphologies in such cases can be ambiguous, cytogenetic findings are not always detected [60], and (to further complicate things) MDS diagnoses can be associated with autoimmune phenomena [61].

In short, the category now called ITP may comprise cases primarily caused by increased platelet destruction, OR by reduced platelet production, OR by some combination of the two. It is difficult to see how progress in understanding the pathogenesis and course of what we now call ITP can be made until separate entities are carved out of this category with specific diagnostic methods.

Parallels between WAS and ITP

As mentioned previously, there is a high rate of ITP in splenectomized WAS patients. Some WAS patients show reduced but not absent leukocyte WASP levels, a variant due in most cases to missense mutations in the first four exons of the gene (previously referred to as X-linked thrombocytopenia). These patients can present with a fluctuating course of thrombocytopenia clinically similar to ITP [62]. Increased uptake of platelets by splenic macrophages is seen in both ITP [63] and WAS [38], although the "foamy" macrophages characteristic of the former have not been reported in the latter. In WAS, platelet uptake has been reported in bone marrow macrophages as well [13]. And as for ITP, both impaired platelet production and increased platelet destruction contribute to the thrombocytopenia of WAS.

One might conclude that WAS patients demonstrate features similar to ITP for the same reasons that they (and WASP(−) mice [64]) develop other autoimmune diseases (at a clinical incidence rate as high as 70% [65]). This predisposition may be only indirectly linked to any intrinsic defect in platelet production or consumption. In this view, impaired platelet production and accelerated baseline platelet consumption leave WAS patients with little ability to maintain a near normal platelet count in response to the stress of even a relatively weak autoimmune state.

An alternative model would hold that rapid platelet consumption and a predisposition toward ITP are two manifestations of the same intrinsic platelet defect. In support of this

"platelet intrinsic" model, our lab has found that the in vivo consumption of WASP(−) platelets is more accelerated by antibody opsonization than is that of WT platelets [17]. Their uptake by bone marrow derived macrophages is similarly increased, at a rate comparable to that of CD47(−/−) platelets. That similarity is significant because the absence of CD47 from platelets or red cells augments, by a well-characterized mechanism, Fc receptor-mediated signal transduction and subsequent phagocytosis. CD47 deficient mice have a mild thrombocytopenia; clearance of their platelets is more accelerated by antibody opsonization than is that of WT platelets; ex vivo phagocytosis of both their platelets and red cells is enhanced; and on the NOD background, they all acquire over time a lethal autoimmune hemolytic anemia [66–68]. These results support the notion that increased phagocytosis of antibody opsonized platelets can both result from autoimmunity and fuel its development.

There is evidence that macrophage function is inhibited by WASP deficiency [69–71], a finding that argues against the above model. But in our hands, this defect is not evident in terms of platelet uptake; WASP(−) macrophages take up opsonized platelets as efficiently as do WT macrophages [17]. Also, the idea of a global impairment of macrophage function in WAS is difficult to reconcile with the high incidence in WAS patients of forms of autoimmunity (AIHA, ITP) which depend on increased phagocytic activity. Impairment of phagocytosis by WASP deficiency in specific cell types and/or contexts seems more likely.

While antiplatelet antibodies have not been rigorously studied in WAS patients, there are several reports of increased levels of platelet-associated antibodies in both WAS [23, 72–74] (although not all reports agree [15]) and ITP [75]. In the case of ITP, however, this finding has a low specificity. In the WAS reports, there is significant variation in methodology, and some of the reports omit control values. Interpretation of these studies is therefore difficult.

There is, however, evidence that WASP(−) mice develop antiplatelet antibodies. On the B6 background, WASP(−) mice show a platelet count about 50% that of WT mice, with a percentage of reticulated platelets comparable to that of WT. About 10% of these mice, however, show both a more significant thrombocytopenia and an elevated percentage of reticulated platelets [17]. This suggests the presence of antiplatelet antibodies. Using an enhanced flow cytometric assay designed to avoid some of the above-mentioned problems associated with platelet targets, we have detected such antibodies in a number of WASP(−) mice, and in no WT mice. An example is shown in Fig. 1. The physiologic significance of one such antibody was evident when we found that it correlated with rapid consumption of CMFDA-labeled WT platelets [17].

If clinical WAS is in fact some form of ITP, one would expect IVIG to be beneficial. In most reported cases, it is not [74, 76]. A possible explanation for this derives from our observation of increased ex vivo phagocytosis, and in vivo clearance, of antibody opsonized WASP(−) platelets. IVIG is thought to inhibit phagocytosis of antibody opsonized platelets. If WASP deficiency inhibits the same antiphagocytic mechanism(s) that are stimulated by IVIG, the latter's effectiveness could be compromised.

Unanswered questions

Because WASP deficiency appears to affect both platelet production rate and consumption rate, a better understanding of the thrombocytopenia of WAS is likely to require progress on both fronts. Concerning megakaryopoiesis, the evidence in favor of a distinct difference between the effect of WASP deficiency in humans versus the mouse model has yet to be

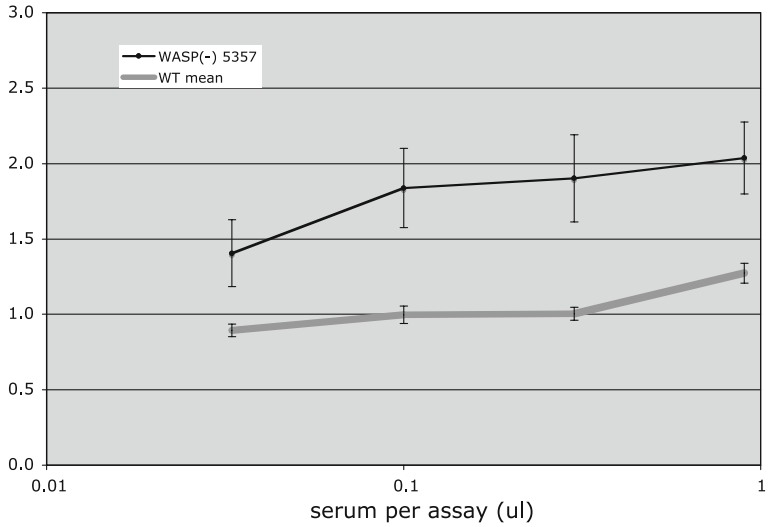

Fig. 1 Detection of anti-platelet antibody in serum from a WASP(−) mouse. Formalin fixed platelets from muMT(−/−) mice were exposed to the indicated amounts of serum from multiple WT males ($n = 12$) and one WASP(−) male (eight repeat assays). Bound antibody was detected with a FITC-labeled anti-IgG/M cocktail and quantified as the geometric mean fluorescence intensity. For each assay, this value was normalized to an internal control (target platelets exposed to no serum). The WASP(−) mouse had an unusually low platelet count (315 K/μl) and elevated RP (25.4%). Results are from a manuscript in preparation (Marathe B, Prislovsky A, and Strom TS, Anti-platelet antibodies in WASP(−) mice induce rapid platelet clearance, 2008, in preparation)

mapped to a specific functional defect. For thrombopoiesis, however, it may be the case that the WASP-dependent amplification of β2-integrin signaling seen in neutrophils [77] is precisely mirrored in the function of $\alpha 2\beta 1$ integrin in megakaryocytes [29]. Evaluation of the activities of specific signal transduction intermediates will determine if that is the case. One might then envision studies of how specific antiplatelet antibodies could affect this or related pathways, whether in the context of WAS or not.

Multiple questions remain in the area of platelet consumption. Does the presence of antiplatelet antibodies correlate with the degree of thrombocytopenia in WASP(−) mice? Do these antibodies have the same differential effect on the in vivo clearance of WASP(−) platelets seen after opsonization with commercially available antibodies? Does that clearance correlate with increased in vivo uptake by macrophages? If so, which ones: splenic, hepatic, bone marrow, lymph node? And if the answers to those questions support the platelet intrinsic model, by what biochemical mechanism might the loss of platelet WASP result in increased platelet uptake by macrophages? We posited that WASP might be necessary to augment the interaction of CD47 with its macrophage receptor, SIRP-alpha, in a manner similar to the role of WASP in augmenting formation of the immunologic synapse. But we have also observed that removing WASP from CD47(−/−) platelets increases the rate at which the latter, after opsonization, are taken up by macrophages ex vivo [17]. This suggests a role for WASP that is distinct from the CD47-SIRP-alpha signal transduction pathway.

Is the "baseline" level of thrombocytopenia in those WASP(−) mice with no evidence of antiplatelet antibodies somehow caused by the same type of increased phagocytosis see with opsonized platelets, or is it due to some other effect of WASP deficiency on, for

example, the rate or quality of thrombopoiesis, or the rate of microparticle formation? The first option is plausible because platelets normally carry a significant amount of surface antibodies which could augment the baseline rate at which they are phagocytosed. The second might explain the small platelet size in clinical WAS, and could open up multiple lines of investigation of the immunogenicity and functional effects of these particles.

These questions also have significant clinical implications. The competitive repopulating advantage of several types of WASP expressing leukocytes, which has been observed both in the context of gene therapy of murine WAS and in spontaneous clinical WAS revertants, makes this condition an appealing candidate for a clinical gene therapy trial. But correction of the platelet count has not been reported in murine gene correction studies [8, 9] or in revertants. There are several plausible explanations for this involving efficiency of gene transfer/expression and the lineage in which reversion occurred. Alternatively, the platelet intrinsic model might suggest that unless all WASP(−) platelets are eliminated, they could continue to promote autoimmunity, and thrombocytopenia, in either a gene correction or spontaneous reversion context.

The platelet intrinsic model for the development of both antiplatelet antibodies and thrombocytopenia could have larger implications as well. A similar mechanism could apply in other cell types in a way that augments and/or promotes the other types of autoimmunity commonly seen in WAS patients. It could also contribute, as autoimmunity is known to do in other contexts, to the development of the lymphoproliferative diseases which are also frequently seen in these patients.

The pathophysiology of WAS, like that of other rare genetic diseases, could end up shedding unexpected light on conditions thought to be unrelated to it. Wiskott may well have foreseen that when, over 70 years ago, he speculated about the still problematic relationship between WAS and ITP.

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