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Evolution and Utility of Antiplatelet Autoantibody Testing in Patients with Immune Thrombocytopenia

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ABSTRACT

To this day, immune thrombocytopenia (ITP) remains a clinical diagnosis made by exclusion of other causes for thrombocytopenia. Reliable detection of platelet autoantibodies would support the clinical diagnosis, but the lack of specificity and sensitivity of the available methods for platelet autoantibody testing limits their value in the diagnostic workup of thrombocytopenia. The introduction of methods for glycoprotein-specific autoantibody detection has improved the specificity of testing and is acceptable for ruling in ITP but not ruling it out as a diagnosis. The sensitivity of these assays varies widely, even between studies using comparable assays. A review of the relevant literature combined with our own laboratory's experience of testing large number of serum and platelet samples makes it clear that this variation can be explained by variations in the characteristics of the tests, including in the glycoprotein-specific monoclonal antibodies, the glycoproteins that are tested, the platelet numbers used in the assay and the cutoff levels for positive and negative results, as well as differences in the tested patient populations. In our opinion, further standardization and optimization of the direct autoantibody detection methods to increase sensitivity without compromising specificity seem possible but will still likely be insufficient to distinguish the often very weak specific autoantibody signals from background signals. Further developments of autoantibody detection methods will therefore be necessary to increase sensitivity to a level acceptable to provide laboratory confirmation of a diagnosis of ITP.

Contents

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Immune thrombocytopenia (ITP) is a benign hematological disorder, which may lead to severe hemorrhagic diathesis, sometimes requiring extensive therapy for many years [1,2]. Although platelet autoantibodies are the major underlying cause for ITP, whether or not to use platelet autoantibody detection for diagnosing ITP has been the subject of discussion for more than 40 years. More recent guidelines do indicate that performing glycoprotein (GP)-specific autoantibody detection may be useful; still, the general tendency remains to diagnose ITP by excluding other causes [3-5]. In this review, we provide a short history and discuss in detail the GP-specific autoantibody methods for autoantibody detection in detail.

For many decades, clinicians have managed without this test, and ITP was confirmed on the basis of a lacking alternative diagnosis for the patient's thrombocytopenia. The leading questions in this review are if platelet autoantibody detection adds value to the diagnostic workup of ITP? In other words, is the specificity and/or sensitivity of the presently available methods sufficient enough to serve as gold standard to diagnose or discard ITP as diagnosis?

The McMaster ITP registry set up in Canada showed that 36 of 295 (12.2%) adult patients initially diagnosed with ITP were found not to have ITP at follow-up and that 10 of 319 (3.1%) patients initially diagnosed with other causes of thrombocytopenia eventually turned out to have ITP [6]. In patients referred to our laboratory for platelet autoantibody detection, we could recently confirm these findings. After analysis of clinical data received several months after the routine requests (ie, independent of the autoantibody detection results), ITP could be excluded for 76 of 165 (46%) patients [7]. Also, the 10-year retrospective medical record review by Bryant et al of a large cohort (n = 492) of children/adolescents (aged 0-18 years) initially diagnosed with ITP showed a different final diagnosis in 14% [8]. Most of these revised ITP diagnoses could well have been diagnosed earlier through a thorough evaluation of the clinical symptoms. However, some needed extensive laboratory investigation before a final diagnosis could be made. In addition, because of the growing availability of large-scale DNA sequence methods, we now see that some patients have been diagnosed with and treated for ITP for many years, but it has now been demonstrated that they suffer from an inherited disorder [9]. Overall, these data show that the sensitivity and specificity of a clinical diagnosis of ITP with "exclusion of other causes" are still not optimal and that more reliable tests for the diagnosis of ITP would be helpful. The importance of good serological testing is evident, of course, while autoantibodies against platelets play a central role in the ITP pathology [2,10]. Although cellular autoimmune responses with T-cell cytotoxicity have been described [11-13] and may also be a cause of ITP, any reliable method for detecting platelet autoantibodies would support clinical diagnosis in the large majority of ITP patients [4].

Historically, some of the first platelet autoantibody detection methods measured the serum-induced platelet-dependent endpoints such as aggregation, lysis, or granule release; and 2-step assays measured platelet-bound and circulating platelet-reactive antibodies, making use of fluorescence-labeled anti–human immunoglobulins (Ig) [14-16]. The 2-step methods with fluorescently labeled anti–human Ig antibodies were a major breakthrough and more reliable than platelet activation assays, but their sensitivity and specificity were still insufficient. Incubation of patient platelets with fluorescent-labeled anti-IgG in the direct (ie, measurement of patient platelet-bound autoantibodies) platelet immunofluorescence test (PIFT) detects platelet autoantibodies in approximately 70%-80% of ITP patients, with a specificity of approximately 50%-60% [16-18]. Nonspecific antibody binding, for example, by immune complexes binding via the platelet-IgG-Fcγ receptor type IIa causes false-positive test results in many non-ITP patients [19,20]. It was first shown by van Leeuwen et al (1981) that a high percentage of autoantibodies in sera from ITP patients reacted positive in the PIFT with healthy donor platelets but did not react with GPIIb/IIIa-deficient platelets from Glanzmann thrombasthenia patients [21]. At that time, to reduce the problem of nonspecific results, solubilization of the platelet membrane and extraction of the membrane proteins, retaining their antigenicity, with nonionic detergents were described at the time [22-25]. Together with the availability of GP-specific monoclonal antibodies (moabs), this led to the development of GP-specific platelet antibody detection methods. After a first experimental approach by Woods et al (1984) with immobilization of GPIIb/IIIa and Ib/IX on microtiterplates, 2 more sensitive methods were introduced, that is, the immunobead assay by McMillan et al (1987) and the monoclonal antibody immobilization of platelet antigens (MAIPA) assay by Kiefel et al (1987) [26-29]. In our laboratory, the direct MAIPA is used for routine diagnostic detection of autoantibodies in ITP patients and shows good specificity (>95%) and reasonable sensitivity (80%) [7], but varying results were found in different studies. In this review, we will discuss our choices in platelet autoantibody assay design and the assay performance.

1. Introduction of GP-Specific Assays for Detection of Platelet Autoantibodies With Increasing Sensitivity of GPIIb/IIIa and GPIb/IX Autoantibody Detection

Following the findings by van Leeuwen et al, Woods et al attached isolated GPIIb/IIIa on microtiter plate wells coated with a GPIIb/IIIa-specific moab, enabling them to confirm the presence of GPIIb/IIIa-specific autoantibodies in plasma from 5 of 56 chronic ITP patients for the first time [27]. In the same year, they showed that GPIIb/IIIa was not the only target for autoantibodies, as 3 of 106 plasmas from chronic ITP patients were reactive with immobilized GPIb [26] . One of these GPIb-reactive samples also reacted with GPIIb/IIIa, suggesting the presence of various specificities of antibodies in patients. Furthermore, in the GPIIb/IIIa and GPIb studies, all 34 and 59 samples, respectively, from patients with a variety of other platelet disorders showed negative results, indicating a high test specificity. Unfortunately, the number of positive samples detected with these early GP-specific assays turned out to be very small. Further optimization of the assay and the idea that free-circulating autoantibodies may be less detectable than platelet-bound autoantibodies became the subject for many follow-up studies. McMillan (1987) introduced moab-coated beads to specifically target GP for the detection of platelet-associated and free-circulating autoantibodies, which proved to be more sensitive [28]. By testing platelet eluates in this technique, platelet-associated autoantibodies were detected in 21 of 28 (75%) ITP patients, whereas free-circulating autoantibodies were detected in 34 of 59 (57.6%) patients. Again, none of the 31 non-ITP thrombocytopenic patients showed reactive autoantibodies. However, for unclear reasons, only 2 of 34 samples responded with both GP, which later turned out to be too low a percentage. The development of the antigen-capture enzyme-linked immunosorbent assay (ACE) and MAIPA allowed for more standardized and reliable platelet antibody detection [29,30]. Autoantibodies were detected in 58 of 81 (72%) sera from suspected ITP patients in MAIPA by Kiefel et al (1991), of which 17 (29%) were GPIIb/IIIa specific, 19 (33%) were GPIb/IX specific, and 22 (38%) were reactive with both GP [31].

An overview of studies using GP-specific methods for the detection of autoantibodies is shown in Table 1. In most studies, approximately 60%-80% of autoantibodies react with GPIIb/IIIa and 50% with GPIb/IX. Most samples contain antibodies with both types of GP specificities, but still, a significant percentage (10%-40%) reacts with only 1 GP (Table 1). These findings indicated that it is necessary to test both GPIIb/IIIa and GPIb/IX for the detection of autoantibodies. However, despite initial reasonable results from McMillan and Kiefel with >70% sensitivity, this percentage was no longer met in subsequent studies, triggering a search for other antibody binding sites and further optimization of the autoantibody detection assays.

Table 1
Autoantibody testing studies in patients with ITP[a, b]

Author	Assay	Material tested	Number of pl used in test[a] × 10^6	Patients	Healthy controls	Non-ITP	GP-specific antibodies						Pat pl counts	Assay cutoff	GP specific MoAB
				Pos/total (%)	Pos/total (%)	Pos/total (%)	GPIIb/IIIa	GPIb/IX	GPIa/IIa	GPV	GPIV	Combi GP			
Woods et al, 1984 [27]	ELISA	Plasma		5/56 (9%)	0/16	0/34	5	nt	nt	nt	nt	nt		Mean controls + 3 SD	2G12 (IIb/IIIa)
Woods et al, 1984 [26]	ELISA	Plasma		3/73 (4%)	0/22	0/59	nt	3	nt	nt	nt	nt		Mean controls + 3 SD	AP1 (Ib)
McMillan et al, 1987 [28]	Immunobead	Eluates		21/28 (75%)		0/15	13/21 (62%)	8/21 (38%)	nt	nt	nt	0/21 (0%)		Mean controls +2SD	2A9 (IIb), 3F5 (IIb/IIIa, 2G12 (IIb/IIIa), P3 (Ib)
		Plasma		34/59 (58%)		0/20	23/34 (68%) 21 (62%) only[b]	13/34 (38%) 11 (32%) only	nt	nt	nt	2/34 (6%)		Mean controls +2SD	2A9 (IIb), 3F5 (IIb/IIIa, 2G12 (IIb/IIIa), P3 (Ib)
Kiefel et al, 1991 [31]	MAIPA	Sera	100	58/81 (72%)			39/58 (67%) 17 (29%) only	41/58 (33%) 19 (33%) only	nt	nt	nt	22/58 (38%)		0.2	Gi5 (IIb/IIIa), FMC25 (IX)
He et al, 1994 [32]	Immunobead	Sera		32/47 (68%)	1/43 (2%)	0/15	22/32 (69%) 5 (23%) only	24/32 (75%) 5 (23%) only	3/32 (9%) 0 (0%) only	nt	12/32 (38%) 2 (6%) only	20/32 (63%)	7-120	Mean controls +2SD	SZ22 (IIb), SZ21 (IIIa), SZ1 (Ib/IX), SZ2 (Ib), Gi6 (Ia/IIa), FA6-152 (IV)
Gaiger et al, 1994 [89]	MAIPA	Platelets	50	14/40 (35%)			14/16 (88%%) 7 (44%) only	9/16 (56%) 2 (13%) only	nt	nt	nt	7/16 (44%)	4-700	Mean controls + 6 SD	VIPL1 (IIb/IIIa), VIPL3 (IIb/IIIa), FMC25 (IX), SZ1 (Ib/IX)
		Sera		5/45 (11%)					nt	nt	nt		4-700	Mean controls + 6 SD	
Hou et al, 1995 [85]	MACE	Platelets	100	30/60 (50%)	0/60		22/30 (73%) 14 (47%) only	16/30 (53%) 8 (27%) only	nt	nt	nt	8/30 (27%)	32 patients <150 28 patients >150	Mean controls + 3 SD	AP1 (Ib), AP2 (IIb/IIIa)
Stockelberg et al, 1996 [88]	MACE	Sera	40	23/65	0/40		12/23 (52%) 8 (35%) only	15/23 (65%) 11 (48%) only	nt	nt	nt	4/23 (17%)	1-463	Mean controls + 3 SD	AP1 (Ib), AP2 (IIb/IIIa)
Brighton et al, 1996 [60]	MAIPA	Platelets	100	40/81 (49%)		11/51 (22%)	35/40 (88%) 19 (48%) only	21/40 (53%) 5 (13%) only	nt	nt	nt	16/40 (40%)	<140	Mean controls + 3 SD	SZ21 (IIIa), SZ22 (IIb), AP2 (IIb/IIIa, AK2 (Ibα), FMC25 (IX)
		Sera	100	23/93 (25%)		2/53 (4%)	16/23 (70%) 7 (30%) only	16/23 (70%) 7 (30%) only	nt	nt	nt	9/23 (39%)	<140	Mean controls + 3 SD	SZ21 (IIIa), SZ22 (IIb), AP2 (IIb/IIIa, AK2 (Ibα), FMC25 (IX)
Crossley et al, 1997 [86]	MAIPA	Platelets	10-100	11/23 (48%)			11/11 (100%)	nt	nt	nt	nt	nt	1-334	0.352	Clone 189/21-10 (IIb/IIIa)
Joutsi and Kekomaki, 1997 [36]	Simult. MAIPA	Platelets	60	46/159 (29%)		Simultaneous MAIPA for GPIIb/IIIa, GPIb/IX and GPIa/IIa								Pat result/mean contr. >1.5	AP1 (Ibα), AP2 (IIb/IIIa), Gi9 (Ia/IIa)
	MAIPA	Platelets	60	13/125 (10%)			Sufficient platelets to also test for GPV					nt		Pat result/mean contr. >1.5	SW16 (V)
Porcelijn et al, 1998 [17]	MAIPA	Platelets	15 (IIb/IIIa) 40 (other	19/47 (40%)		5/26 (19%)	11/19 (58%)	8/19 (42%)	0/19	12/19 (63%)	0/19	6/19 (32%)	<100	0.3	C17 (IIb/IIIa), MB45 (Ibα), 10G11 (Ia/IIa), SW16 (V), P58 (IV)

			GP)											
Warner et al, 1999 [61]	MAIPA	Platelets		19/49 (39%)		3/32 (9%)	19/19 (100%)	nt	nt	nt	nt	nt		0.2	Raj-1 (IIb/IIIa)
	Antigen capture assay	Platelets		19/49 (39%)		3/32 (9%)	19/19 (100%)	nt	nt	nt	nt	nt		0.2	Raj-1 (IIb/IIIa)
	Antigen capture assay	Platelets		37/56 (66%)		2/26 (8%)	31/37 (84%) 8 (22%) only	28/37 (76%) 5 (14%) only	nt	nt	nt	23/37 (62%)		0.2	Raj-1 (IIb/IIIa), TW-1 (Ib)
Kosugi et al, 2001 [98]	MACE	Eluates	50	18/47 (38%)			18/18 (100%)	nt	nt	nt	nt	nt		Mean controls + 3 SD	AP3 (IIIa)
Fabris et al, 2002 [99]	Commercial MACE	Platelets		39/65 (60%)		1/39 (3%)	29/39 (74%)	31/39 (79%)	0-21/39 not specified	nt	nt	21/39 (54%)	<100	Mean controls + 3 SD	AP2 (IIb/IIIa), 142.1 (Ib/IX), 143.1 (Ia/IIa)
Chan et al, 2003 [100]	MACE	Platelets		43/59 (73%)	0/30	5/31 (16%)	43/43 (100%)	nt	nt	nt	nt	nt		OD 0.200	Raj-1 (IIb/IIIa)
Fabris et al, 2004 [101]	Commercial MACE	Platelets		25/50 (50%)			18/25 (72%)	15/25 (60%)	2/25 (8%)			8/25 (32%)	< 100		
Davoren et al, 2005 [87]	Commercial ELISA	Eluates		114/216 (53%)		13/46 (28%)	95/114 (83%) 14 (12%) only	84/114 (74%) 3 (3%) only	94/114 (82%) 7 (6%) only			90/114 (79%)	1-834		
Tomer et al, 2005 [102]	Immunobead Flow	Platelets	100	53/62 (86%)	0/60	0/14	53/62 (86%)	nt	nt	nt	nt	nt	3-166	Ratio pat/3 hc > 1.3	P2 (IIb/IIIa)
Meyer et al, 2006a [94]	MAIPA	Platelets	20	7/8 (88%)			7/7 (100%)	5/7 (71%)	3/7 (43%)	nt	nt	5/7 (71%)		0.2	P2 (IIb/IIIa), FMC25 (IX), Gi9 (Ia/IIa)
		Sera	20	30/33 (91%)			24/30 (80%)	25/30 (83%)	12/30 (40%)	nt	nt	19/30 (63%)		0.2	P2 (IIb/IIIa), FMC25 (IX), Gi9 (Ia/IIa)
	ASPA	Platelets	20	7/8 (88%)			7/7 (100%)	5/7 (71%)	1/7 (14%)	nt	nt	5/7 (71%)			P2 (IIb/IIIa), SZ1 (Ib/IX), AK7 (Ia/IIa)
		Sera	20	32/33 (97%)	0/100		25/32 (78%)	29/32 (91%)	13/32 (41%)	nt	nt	23/32 ((72%)			
Meyer et al, 2006 [103]	MAIPA	Platelets	20	14/28 (50%)			12/14 (86%)	11/14 (79%)	5/14 (36%)	nt	nt	9/14 (64%)		0.2	P2 (IIb/IIIa), FMC25 (IX), Gi9 (Ia/IIa)
	MAIPA	Sera	20	7/39 (18%)			4/7 (57%)	3/7 (43%)	0/7	nt	nt	0/7		0.2	P2 (IIb/IIIa), FMC25 (IX), Gi9 (Ia/IIa)
	Antigen-specific capture assay (ASCA)	Sera	20	30/39 (77%)	0/70		25/30 (83%)	19/30 (63%)	9/30 (30%)	nt	nt	17/30 (57%)			P2 (IIb/IIIa), FMC25 (Ib/IX), AK7 (Ia/IIa)
Panzer et al, 2007 [90]	MAIPA	Platelets		14/40 (35%)			12/14 (86%) 5 (36%) only	9/14 (64%) 2 (14%) only	nt	nt	nt	7/14 (50%)	3-223		
Najaoui et al, 2012 [91]	MAIPA	Platelets	20	129/240 (54%)			104/129 (81%) 41 (39%) only	88/129 (68%) 25 (24%) only	nt	nt	nt	63/129 (49%)		0.15	Gi5 (IIb/IIIa), FMC25 (IX)
He et al, 2013 [104]	MAIPA	Platelets	40	22/50 (44%)	20/86 (23%)		22/22 (100%)		nt	nt	nt	nt	<100	0.470-0.680 depending on moab	SZ21 (IIIa), SZ22 (IIb), SZ1 (Ib/IX), SZ2 (Ib)
Porcelijn et al, 2018 [7]	MAIPA	Platelets	15 (IIb/IIIa) 40 (Ib/IX, V)	51/60 (85%)	1/462 (<1%)	0/43	40/51 (78%) 6 (12%) only	36/51 (71%) 4 (8%) only	nt	31/51 (61%) 3 (6%) only	nt	38/51 (75%)	<100	0.13	C17 (IIb/IIIa), MB45 (Ibα), SW16 (V)
	MAIPA	Platelets	40	13/32			Sufficient platelets to also test for GPIa/IIa					13/13 (100%)	<100	0.13	10G11 (Ia/IIa)
	MAIPA	Platelets	40	Jul-26			Sufficient platelets to also test for GPIV					7/7	<100	0.13	P58 (IV)

(continued on next page)

Table 1 *(continued)*

Author	Assay	Material tested	Number of pl used in test[a] $\times 10^6$	Patients	Healthy controls	Non-ITP	GP-specific antibodies						Pat pl counts	Assay cutoff	GP specific MoAB
				Pos/total (%)	Pos/total (%)	Pos/total (%)	GPIIb/IIIa	GPIb/IX	GPIa/IIa	GPV	GPIV	Combi GP			
Vollenberg et al, 2019 [38]	MAIPA	Platelets	100	343/1140 (30%)			242/343 (71%) 71 (21%)only IIb/IIIa	232/343 (68%) 30 (9%) only Ib/IX	nt	222/343 (65%) 10 (3%) only V	nt	232/343 (68%) (100%)		0.2	Gi5 (IIb/IIIa, FMC25 (IX), SW16 (V)
Al-Samkari et al, 2020 [33]	Commercial ELISA	Eluates		205/228 (90%)		24/108 (22%)	269/280[a] (96%) 48 (17%) only IIb/IIIa	232/280 (83%) 11 (4%) only Ib/IX	145/280 (52%) 0 (0%) only Ia/Ia	nt	nt	221/280 (79%)	1-657		

Empty cells: no data are available in publication. nt, not tested.

[a] Number of platelets used in test: the number of platelets used per GP specificity to test the binding of GP-specific autoantibodies.

[b] Only: meaning only reactive with this GP, not with the other tested GPs.

2. Other GPs as Targets for Autoantibodies and the Impact of GPV-Specific Platelet Autoantibodies

In search of a more accessible autoantibody detection assay on the one hand and better sensitivity on the other hand, research was conducted using different methods into antibody binding to GPIa/IIa, GPIV, and GPV.

He et al (1994) used an immunobead assay to detect autoantibodies in sera of ITP patients not only against GPIIb/IIIa and GPIb/IX but also against GPIa/IIa and GPIV [32]. Autoantibodies reactive with GPIa/IIa or GPIV were detected in 3 (9%) of 47 sera and 12 (38%) sera. None of the sera was only positive for anti-GPIa/IIa, and 2 (6%) of the sera reacted only with GPIV. More recent studies by Porcelijn et al [7] using the direct MAIPA and by Al-Samkari et al [33] using a commercial GP-specific enzyme-linked immunosorbent assay (ELISA) (PAKAuto) confirmed the almost nonoccurrence of autoantibody binding exclusively to GPIa/IIa or GPIV.

GPV as a target for autoantibodies was first reported by Beardsley (1988) in a case of childhood ITP [34]. In 1993, Meenaghan showed that most GP-reactive antibodies in multitransfused patients with bone marrow (BM) failure (also) reacted with GPV [35].

The first study investigating whether platelet-associated autoantibodies in adult ITP patients were also reactive with GPV was conducted by Joutsi et al (1997) [36]. For those patients for whom sufficient platelets could be isolated, GPV reactivity was tested after performing a simultaneous direct MAIPA for GPIIb/IIIa, GPIb/IX, and GPIa/IIa. Thirteen of 125 patients (10%) showed anti-GPV antibodies. In a follow-up study in 69 thrombocytopenia patients with strong reactive autoantibodies in the direct PIFT, they detected anti-GPV in 15 (22%) patients [37]. We (Porcelijn et al, 1998) detected GPV-associated autoantibodies in samples from 12 (63%, 6 specific and 6 in combination with GPIIb/IIIa and GPIb/IX) of 19 ITP patients with positive direct MAIPA results [17]. More recently, after optimization of the direct MAIPA, we detected platelet-associated autoantibodies in 51 of 60 (85%) well-categorized untreated ITP patients, of which 31 (61%) reacted positive with GPV [7]. The major role for GPV-associated autoantibodies in the pathogenesis of ITP was also confirmed by Vollenberg et al (2019) [38]. In their study, platelet-associated autoantibodies were detected in 343 of 1140 (30%) patients suspected for ITP, 242 (71%) positive for anti-GPIIb/IIIa, 232 (68%) positive for anti-GPIb/IX, and 222 (65%) positive for anti-GPV. For 10 (2.9%) samples, only anti-GPV antibodies were detected.

In a cohort of 754 patients, referred to our laboratory for platelet autoantibody investigation, with positive direct MAIPA results (unpublished data), 625 (83%) were positive for anti-GPV, 481 (64%) for anti-GPIb/IX, and 340 (45%) for anti-GPIIb/IIIa. For 178/754 (24%) patients, only GPV-associated autoantibodies were detected. The high percentage of anti-GPV might partly be due to the MAIPA settings, as we see higher average optical density (OD) values for GPV compared with GPIb/IX and GPIIb/IIIa (Fig. 1), which is in contrast to what was seen by Vollenberg et al [38].

Considering these results and the limited number of available patient platelets, we have decided to include GPIIb/IIIa, GPIb/IX, and GPV but not GPIa/IIa and GPIV in our routine diagnostic autoantibody detection protocol.

3. GP-Specific Autoantibody Binding Causing Loss of Platelet Function

The possibility to detect GP-specific autoantibodies was not only a step forward in increasing the specificity of the detection of autoantibodies as a cause for platelet destruction but could also be used to clarify some rarely encountered primary clotting disorders, which were thought to be caused by platelet function loss. These cases were shown to be based on blocking of functional binding sites at the different GPs. First case reports of so-called acquired Glanzmann

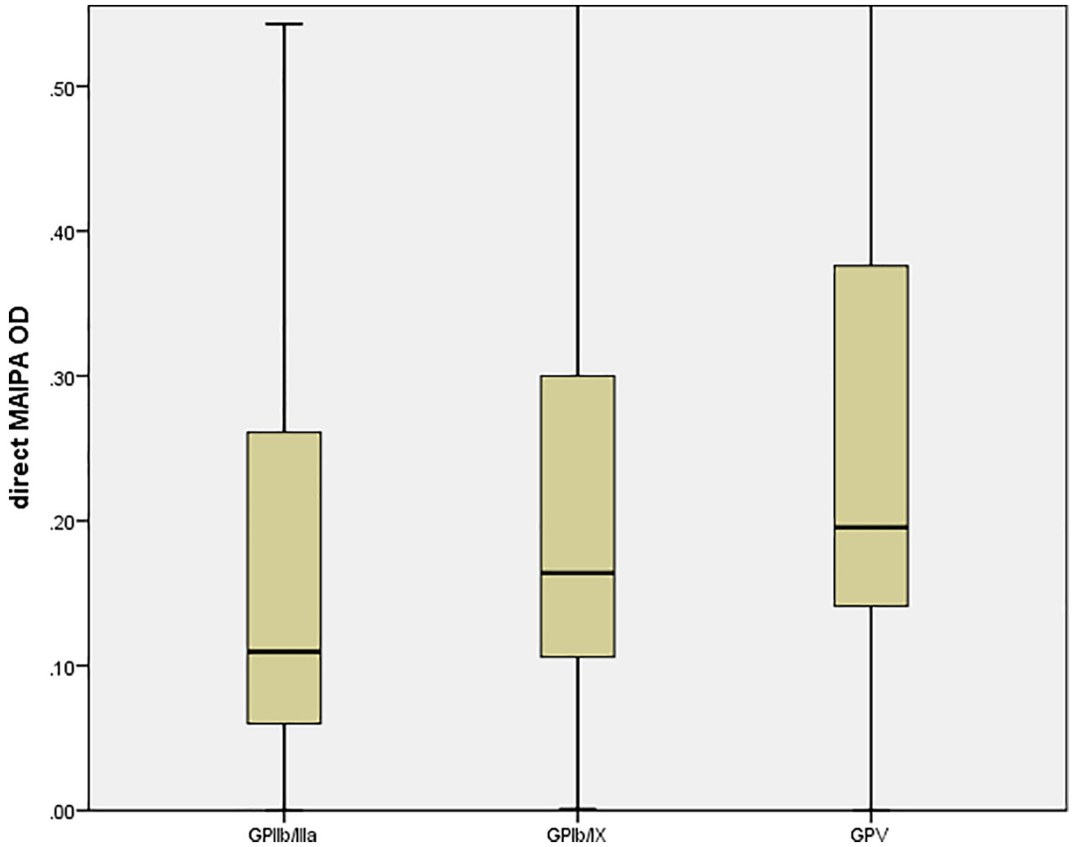

Fig. 1. Routine autoantibody detection with positive results (ie, at least 1 of the tested GP shows positive results) in direct MAIPA (n = 754). For 754 positive direct MAIPA results (ie, at least 1 of the GPs IIb/IIIa, Ib/IX, or V shows OD values >0.130), the results for the different GP are depicted in the boxplots. Remarkable is the difference in mean OD levels between the different GP. This is probably the result of test specifics as, for reasons explained in the text, we use 15×10^6 platelets for GPIIb/IIIa and 40×10^6 platelets for GPIb/IX and GPV. The effect of raising the platelet number for GPIIb/IIIa is shown in Fig. 4.

disorder and acquired Bernard Soulier syndrome were already published in 1987 by Niessner et al and Devine et al, respectively [39,40]. Depending on the specific binding sites on GPIIb/IIIa or GPIb, the autoantibodies may inhibit GPIIb/IIIa-fibrinogen binding, leading to a condition resembling Glanzmann thrombasthenia, a genetic disorder causing GPIIb/IIIa deficiency, or inhibit GPIb–von Willebrand factor binding resembling Bernard Soulier syndrome, which is a genetic disorder causing GPIb/IX/V deficiency. A case of severe impaired response of platelets to collagen due to GPIa/IIa-specific autoantibodies blocking the collagen receptor has also been described by Deckmyn et al (1990) [41]. Interesting in these cases were the often normal platelet counts despite the presence of autoantibodies. This could be explained by either the IgG antibodies being of the IgG2 or IgG4 subclass and subsequently less Fc-Fcγ-receptor binding on macrophages or splenectomy preventing destruction of opsonized platelets [42,43]. We questioned whether the blocking effect of autoantibodies on platelet function also plays a role in ITP patients, leading to a bleeding tendency not only due to thrombocytopenia but also due to a loss of function. We therefore developed a flow cytometry test for measuring platelet aggregation, in which 10- to 25-fold lower platelet counts were necessary than in the routine aggregation assays in an aggregometer [44,45]. Indeed, a decreased platelet aggregation potential in both adult and pediatric ITP patients with GPIIb/IIIa-specific autoantibodies could be demonstrated. To what extent the influence of blocking autoantibodies plays a role in the bleeding tendency in ITP patients is still unknown. More research is needed to objectify the clinical impact of this mechanism. Depending on the results of this research, it is conceivable that this aspect could be included in the treatment of patients with ITP.

4. GPIb-Specific Autoantibodies, Thrombopoietin Production, and Fc-Independent Platelet Destruction

Regulation of platelet production depends on the levels of hematopoietic growth factor thrombopoietin (Tpo). Tpo, mainly produced in the liver, binds to the c-mpl-receptors on CD34+ stem cells and stimulates platelet production [46,47]. In previous studies, we showed that plasma Tpo levels are useful to discriminate thrombocytopenia caused by megakaryocyte and platelet production failure (highly elevated Tpo levels) from thrombocytopenia caused by elevated platelet destruction as in ITP and fetal/neonatal alloimmune thrombocytopenia (normal or only slightly elevated Tpo levels) [17,48,49].

After initial reports in which the presence of GPIb/IX-specific autoantibodies was associated with a diminished response to intravenous immunoglobulin IgG (IVIg) therapy in ITP, several possible mechanisms explaining this observation have been studied [50,51]. It was shown that desialylation of GPIb on senescent platelets triggers removal from circulation by the Ashwell-Morrell receptor (AMR) expressed on hepatocytes in the liver [52,53]. Subsequently, it was demonstrated in mouse models that desialylation of GPIb also occurred after binding of moab specific for the ligand binding domain of GPIbα, causing platelet destruction via the AMR [54]. This so-called Fc-independent platelet destruction route was thought to be a possible explanation for the lesser response on IVIg. Thereafter, Quach et al found that, under shear conditions, binding of moab to the ligand binding domain of GPIbα can exert a pulling force causing activation of GPIb/IX, which can induce Fc-independent platelet clearance [55]. However, in both studies of Al-Samkari et al and Rogier et al, the correlation between the clinical effectiveness of IVIg treatment and the presence of platelet-associated GPIb/IX autoantibodies could not be confirmed [33,56].

More recently, Xu et al described a novel mechanism, in other words, GPIb directly inducing TPO production in hepatocytes [57]. In their mouse model, GPIb-specific moab caused inhibition of TPO production. We measured free plasma TPO levels in a large cohort of patients with positive autoantibody detection in the direct MAIPA and did not find a correlation between antibody GP specificity and free plasma TPO levels [33,58]. These conflicting results require further investigation into the influence of, in particular, GPIb/IX-specific platelet autoantibody binding on TPO production and on the Fc-independent platelet destruction pathway in humans.

5. The Impact of the Change in ITP Definition to <100 Instead of <150 × 10^9/L

For several reasons, the platelet count of the patient is important in the detection of autoantibodies. First, to be able to make an initial classification between patients with possible ITP and patients for whom another underlying cause for the thrombocytopenia is more likely. For instance, in our laboratory, we frequently receive autoantibody requests for pregnant women with platelet counts between 100 and 150 × 10^9/L. As in pregnancy, a physiological drop in platelet count is often seen. Unsurprisingly, the test results for these cases are consistently negative [59]. Second, the sensitivity of the autoantibody detection assays seems inversely correlated with the patients' platelet count. In 1996, Brighton already showed a nonstatistically significant trend toward higher positivity in direct MAIPA for ITP patients with lower platelet counts [60]. This was also seen in the prospective study by Warner et al (1999), in which the GP-specific antigen capture assay was negative for ITP patients with platelet counts >100 × 10^9/L [61]. Third, increased platelet counts as a result of therapy aimed at reducing the number of antibodies will of course reduce the number of positive test results. Indeed, in 170 known ITP patients, categorized in 4 platelet count groups, detection of autoantibodies became less sensitive for patients with platelet counts greater than 100 × 10^9/L (data not shown). In our routine diagnostic setting, we advise clinicians to request autoantibody detection if the platelet count is between 10 (if <10 insufficient platelets can be isolated for direct testing) and 80 × 10^9/L.

6. The Impact of the GP-Specific Monoclonal Antibodies Used in the Assay on the Test Results

Using GP-specific mouse anti-human moabs that are known not to bind to restricted areas targeted by patient autoantibodies is very important to prevent displacement of the latter from the platelet antigens.

Kiefel et al (1991) showed for 3 ITP patients with autoantibodies against GPIb/IX that these antibodies were partially blocked by moab Gi10, directed against a fragment consisting of a part of GPIbα (after protease treatment) and GPIbβ [31]. He et al (1995) showed for 6 of 16 anti-GPIb/IX antibodies specificity for the N-terminal glycocalicin part of GPIbα [62]. These authors were able to specify the main autoepitope for these 6 antibodies to the short fragment AA326-346. We found a significant correlation between the indirect and direct MAIPA OD values for the antibodies directed against GPIIb/IIIa, GPV, and GPIa/IIa but noticed a deviating pattern in patients with strong autoantibodies against GPIb/IX (unpublished data; Fig. 2). We therefore periodically tested the presence of free-circulating autoantibodies using moab MB45 (glycocalicin part of GPIbα) and moab FMC25 (GPIX). All positive results, either with MB45 or FMC25, are shown in Fig. 3. For most samples, indirect MAIPA OD values were higher with FMC25. This is probably caused by autoepitope loss and/or moab binding epitope loss as also shown by Kiefel et al and He et al [31,62]. Intriguing is that we do not see this deviating pattern for GPIb/IX if MB45 is used in the direct MAIPA. In the direct MAIPA, anti-GPIb/IX does not seem to prevent MB45 from binding and vice versa MB45 does not seem to displace the autoantibodies. Because we use frozen (−196°C) platelets for the indirect MAIPA, we also investigated whether the freeze-storage-thaw procedure affects the MAIPA results, for example, by degradation of the glycocalicin part of GPIbα. This did not solve the problem, and further investigation into the exact mechanism causing this discrepancy is necessary. These results again support the importance of carefully selecting the moab for antigen binding in GP-specific assays.

Although varying results were found for autoepitope localization on GPIIb/IIIa, the epitopes for a high percentage of autoantibodies seem to be restricted to some specific areas depending on an intact heterodimeric complex structure [63-65]. Several studies have indicated that a significant percentage of GPIIb/IIIa-reactive autoantibodies actually bind to GPIIb. In 1983, Varon and Karpatkin already noticed a decreased binding of the GPIIb-specific moab 3B2 on platelets from ITP patients [66]. After a first experiment by McMillan et al (2001), observing that autoantibodies from ITP patients reacted with αIIbβ3 but not with αvβ3 expressed on Chinese ovary cells, a more specific antibody-binding localization between the amino acids L1 and Q449 of the N-terminal half of the β-propeller domain in αIIb was shown (McMillan et al, 2002) [67,68]. This restricted region was confirmed by Kiyomizu et al (2012) and mapped to specific loops and critical amino acids in this region [69]. Restricted locations for autoantibody binding were also noticed using anti-GPIIb/IIIa F(ab′)2 fragments from 2 ITP patients and Fab fragments from 2 human monoclonal anti-GPIIb/IIIa, both inhibiting the binding of anti-GPIIb/IIIa from other ITP patients [70-72] . The restricted binding of platelet antibodies is further supported by IgG light chain restriction and limited numbers of B-cell clones producing autoantibodies in ITP patients [73,74].

In 2012, the Scientific Subcommittee of the International Society on Thrombosis and Haemostasis recommended to use moab to each of the GPIIb (eg, SZ22) and GPIIIa (eg, SZ21) subunits or to the intact GPIIb/IIIa (eg, Gi5, AP2, Raj-1) and GPIb/IX (eg, the GPIbα-specific AP1 or the GPIX-specific FMC25) [75]. In our hands, C17 (GPIIb/IIIa), SW16 (GPV), and FMC25 (GPIX) replacing MB45 (GPIbα) shows best results in the MAIPA.

7. The Impact of Cutoff Values in the Assay

To differentiate specific signals from the noise, the assay cutoff value to be used is, of course, very dependent on the test specifics. For example, longer incubation steps in the "2-day" MAIPA [29] for autoantibody detection, in comparison with the "1-day" MAIPA [76] which we use for HPA alloantibody detection, give better signals to noise ratios for the often weakly reactive autoantibodies. Reported assay cutoff values to determine positive vs negative results vary among articles even when comparable MAIPA assays are used. The essence of choosing specific ODs of course is to have the best (trade of between) sensitivity and specificity which should, respectively, be validated by true ITP patients and true non-ITP patients with varying platelet numbers. By testing a large group of healthy subjects and non-ITP thrombocytopenia patients, we were able to set the cutoff value to OD = 0.13 (mean 462 healthy controls + 3 SD) without compromising specificity [7]. The difference between 0.130 and the often used 0.200 seems only minimal, but in a series of 754 routine request samples with at least 1 of the GPs IIb/IIIa, Ib/IX, or V reacting positively in direct MAIPA, the highest OD was only between 0.130 and 0.200 for 273 (36%) samples (unpublished data). The importance of having low background signals can also be seen in Fig. 2. Remarkably, correlation between direct and indirect MAIPA results can still be observed for OD levels between 0.050 and 0.130, indicating specific autoantibody signals even with very low OD values that would be classified as negative. These results could mean that, especially for the indirect MAIPA, the cutoff value of the mean of healthy controls +3 SD is still too high to sensitively detect platelet autoantibodies. Illustrating in this context is the high sensitivity of 90% at the expense of a lower specificity (78%), found by Al-Samkari et al when testing platelet eluates of suspected ITP patients in the commercial PAKAuto assay [33].

Therefore, we can conclude that with the available GP-specific assays, without compromising specificity, an acceptable sensitivity for

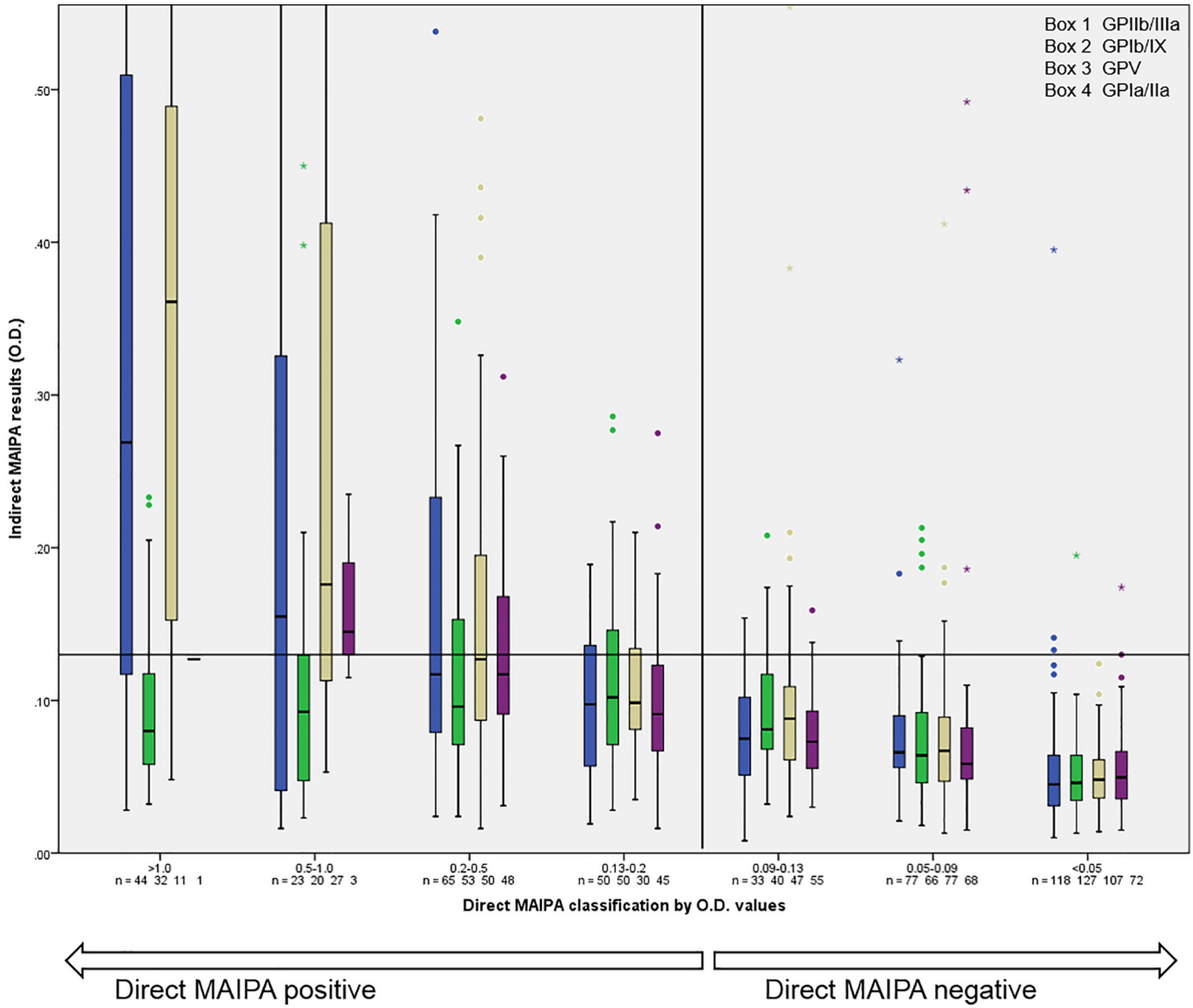

Fig. 2. Indirect vs direct autoantibody detection with MAIPA (n = 400). Comparing direct (platelet-associated, groups categorized in OD ranges) and indirect (free-circulating) MAIPA results shows a highly significant correlation. Interesting is the correlation continuing below the cutoff level of 0.130 (indicated by lines). Remarkable is the deviating correlation between the strongly reactive direct and less reactive indirect GPIX MAIPA results. For this reason (see text), we decided to change from moab MB45 (GPIbα) to moab FMC25 (GPIX) (see Fig. 3).

ruling out ITP will not be possible and other methods will be necessary to distinguish background from noise.

8. The Impact of Autoantibodies on Platelet Production

It must be emphasized that ITP is a disorder causing not only increased platelet destruction but also decreased platelet production. GPs are already expressed on megakaryocytes (MKs) during maturation [77], and GPIIb/IIIa, GPIb/IX, and GPIa/IIa autoantibodies are known to cause inhibition of MK maturation as well as proplatelet and platelet formation [78,79]. Although most ITP patients show normal MK numbers in the BM, Houwerzijl et al (2006) found MK in ITP patients having characteristics of apoptosis-like programmed cell death [80]. Lev et al and Grodzielski et al (2018) studied the interference of autoantibodies with the MK binding to their ligands [81,82]. Anti-GPIa/IIa antibodies caused a decrease in adhesion of GPIa/IIa to collagen I and a decrease in phosphor-MLC2 levels, leading in the early phase of MK maturation, in the osteoblast niche, to premature platelet release. Anti-GPIIb/IIIa and anti-GPIb/IX interfere with the MK-fibrinogen, respectively, MK-von Willebrand factor interaction, leading to functional abnormalities and inhibited proplatelet production.

Shestra et al (2020) hypothesized that a percentage of autoantibodies might be sequestered in the BM, targeting platelet progenitor cells and newly produced platelets, which could be one of the reasons for the absence of detectable autoantibodies in peripheral blood [83]. They investigated the presence of autoantibodies in BM, testing cell-free BM fluid and a mixture of mononuclear cells, platelets, and MK for the presence of GPIIb/IIIa and GPIb/IX autoantibodies, in the indirect, respectively, direct antigen capture assay. Seven of 18 (39%) patients had detectable antibodies in the direct ACE and 3 (17%) in the indirect ACE. Five of 10 patients showed detectable antibodies in the BM, which could not be detected in the peripheral blood. All controls, that is, healthy controls (n = 6) and non-ITP thrombocytopenic patients (n = 3), had no detectable autoantibodies in the BM. BM testing increased the sensitivity for autoantibody detection with ACE from 60% to 72%.

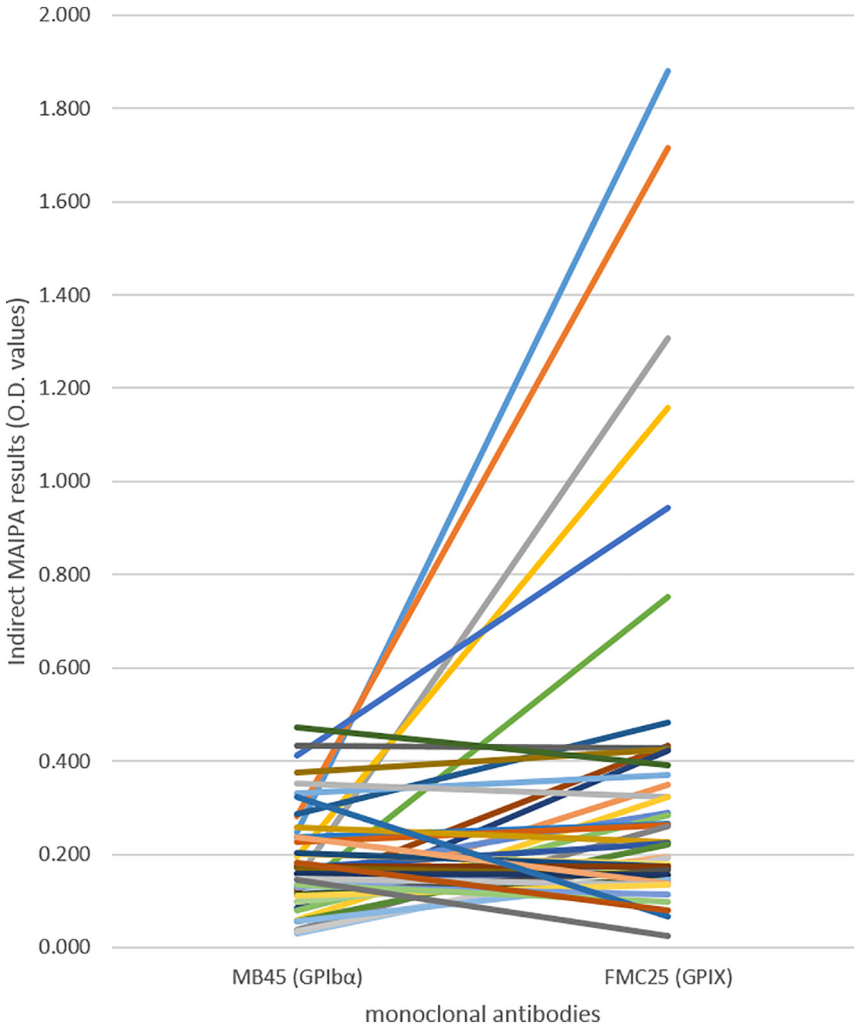

Fig. 3. Comparison anti-GPIb/IX positive indirect MAIPA results if using the moab MB45 or FMC25 (n = 45). The indirect MAIPA results, using MB45 and FMC25 for 45 positive results (ie, positive with MB45 and/or positive with FMC25), are shown.

9. Discussion

We would like to emphasize that autoantibody detection can make a valuable contribution to diagnosing ITP. Recently, Vrbensky et al (2019) published a systematic review and meta-analysis of platelet autoantibody tests in the diagnosis of ITP in which 18 eligible studies were included [4]. They concluded that autoantibody testing in ITP patients has a high specificity but a low sensitivity and that a positive autoantibody test result can be useful for ruling in ITP but a negative test result does not rule out ITP. Notably, we have recently performed a systematic review of platelet autoantibody assays in childhood ITP and reached a more or less similar conclusion for autoantibody detection [84].

Now, we have revisited the 18 studies included in the article of Vrbensky et al and completed the series with the 4 studies introducing GP-specific assays [26-28,31] and 9 studies also giving information on the GP specificity of platelet autoantibodies, including the recent articles by Vollenberg et al and Al-Samkari et al [17,32,36,85-88]. To understand the significant differences in sensitivity found in the studies, we compared some important aspects. First, 5 of 9 eligible studies used by Vrbensky et al for the calculation of the sensitivity only tested for GPIIb/IIIa and GPIb/IX [60,61,89-91]. Three also for GPIa/IIa [92-94], and only 1 also tested for GPV [7]. As was recently confirmed, GPV is an important target for autoantibodies [38,95]. The exact increase in sensitivity by including GPV probably very much depends on the test characteristics. For instance, Vollenberg et al did not see any differences in antibody load for the different GPs [38], which triggered us to investigate the GP-specific OD values for the direct MAIPA positive results. In our series, GPV shows higher OD levels than GPIb/IX and GPIIb/IIIa (Fig. 1). Importantly, if GPV would not have been included in our routine setting, we would have missed 178 of 754 (24%) positive results. In addition, we considered that the relatively low GPIIb/IIIa OD values might be the result of a difference in platelet numbers used in the direct MAIPA because the expression of GPIIb/IIIa is higher than the other GPs. For optimal use of the limited patient platelets available for testing, we use 15×10^6 platelets in the MAIPA for GPIIb/IIIa vs 40×10^6 for GPIb/IX and for GPV. The effect of increasing the input from 15 to 40×10^6 platelets per test is shown in Fig. 4. These results made us decide to increase the platelet numbers used in the indirect MAIPA for the detection of GPIIb/IIIa-reactive autoantibodies to 40×10^6 per test. As shown in Table 1, the platelet numbers used for testing vary significantly between studies, which also complicate comparing the results. Second, patients with platelet counts $>100 \times 10^9$/L were included in 3 [60,89,90] of the 9 studies, and platelet counts were not mentioned in 3 other studies [61,91,94]. In our opinion, it is important to limit testing to the group of patients with platelet counts less than 100 or better still less than 80×10^9/L (see below). Third, the low sensitivity of autoantibody detection is mainly due to insufficient signal to noise ratios. In our routine series, 36% (273/754 positive results) showed OD values between 0.130 and 0.200. Most studies used mean of healthy controls (hc) + 3 SD or 0.200 as cutoff value [60,61,89,94]. The mean of hc is,

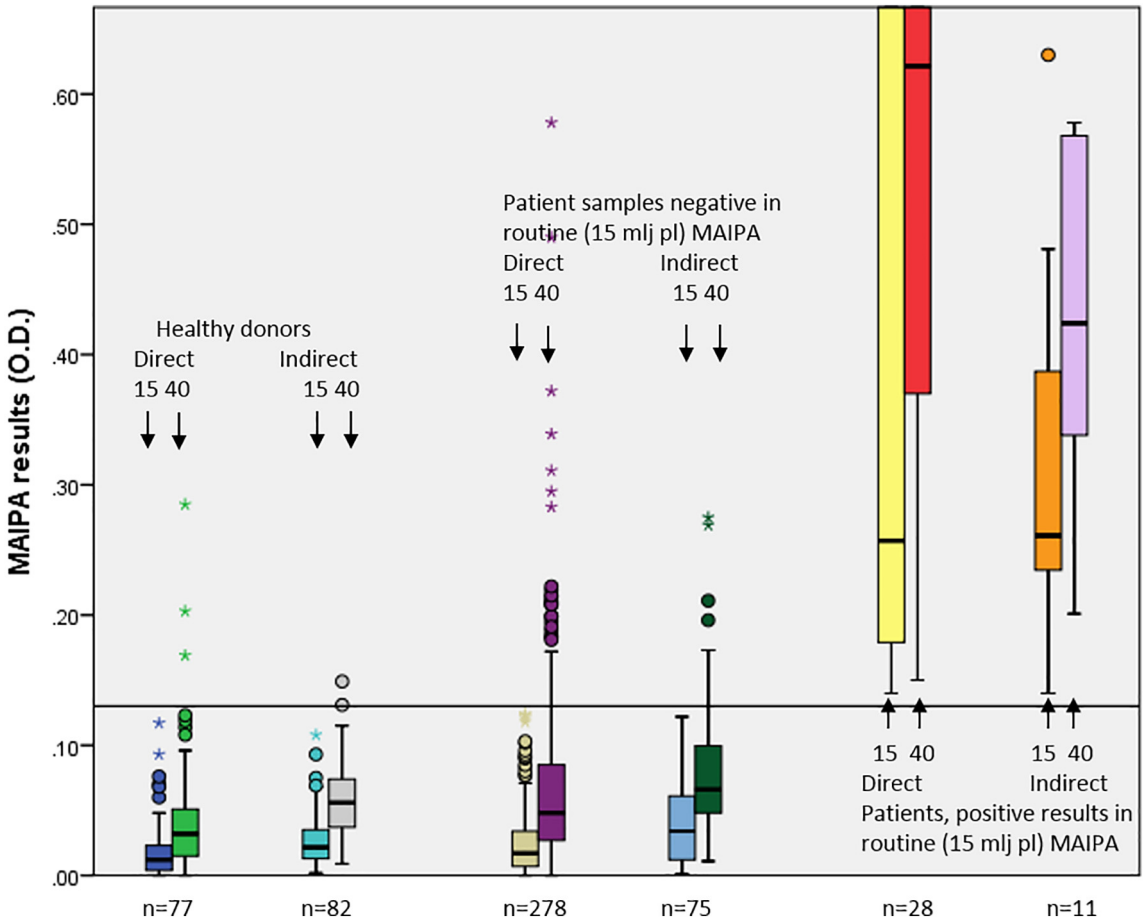

Fig. 4. Fifteen or 40×10^6 platelets used in MAIPA for detection of GPIIb/IIIa reactive autoantibodies. The effect of increasing the number of platelet from 15 to 40×10^6 used in the direct and indirect MAIPA is shown.

of course, dependent on the background signals and varies significantly between studies. Using hc + 3 SD can compromise sensitivity in case of high background signals.

Finally, detection of free-circulating autoantibodies in all available assays is less sensitive than detection of platelet-associated antibodies. This is somewhat surprising conceptually because, after transfusing a platelet concentrate to ITP patients, the 1-hour increment is often zero, indicating that platelets are almost instantly opsonized and removed from circulation. One explanation could be that antibody production, platelet opsonization, and removal occur locally in the spleen and free-circulating autoantibodies in the peripheral blood are less detectable. Considering that direct and indirect autoantibody test results (Fig. 2) correlate even below our cutoff level of 0.130, one could assume that for a percentage of patients (especially free-circulating) autoantibodies are present but are simply too weak to be detected. More sensitive methods will be necessary to detect these antibodies.

In contrast to what was found by Al-Samkari et al (2020) [33] using testing of platelet eluates in the PAKAuto (Immucor), the correlation between circulating and platelet-associated autoantibodies is highly significant in our MAIPA assay (Fig. 2). These different results might well be caused by the different assays used. In the PAKAuto, GPs are already isolated and bound to the microtiterplate wells, whereas in MAIPA, intact platelets are used. The number of GPs per well may vary significantly in PAKAuto but is reasonably comparable between indirect and direct MAIPA using the same number of platelets.

In addition to the detection of GP-specific autoantibodies being of value for the diagnosis of ITP, it is interesting to zoom in on the usefulness of monitoring autoantibodies during treatment and whether the GP specificity of the autoantibodies can be valuable for choice of treatment. We performed serial antibody detection for patients treated with rituximab and found a strong correlation between platelet counts and direct MAIPA OD values [96,97]. Al-Samkari et al also showed a strong correlation between the absence of detectable platelet-associated autoantibodies and clinical remission (sensitivity 87%, specificity 90%) and argued that test results can help clinicians in their choice of treatment [33]. Indeed, knowing that there is a strong correlation between test results and the effect of treatment (ie, platelet counts), serial testing can be supportive for treatment policy, especially for patients with more possible causes for thrombocytopenia. The benefit of knowing the GP specificity of the antibodies is something that needs further investigation. The presence of strong GPIIb/IIIa reactive autoantibodies, possibly causing inhibition of fibrinogen binding, can be a reason to opt for treatment that reduces antibody production rather than for treatment to reduce platelet destruction. Before such choices can be made, we need to better understand the effect of treatment (eg, splenectomy and Tpo) on antibody production. Studying well-categorized ITP patients using reliable GP-specific autoantibody detection methods for serial testing during treatment will hopefully provide more insight in the near future. The interaction between the Fc-independent platelet destruction pathway via the AMR, Tpo production, GPIbα desialylation, and platelet autoantibody specificity is intriguing, but varying and sometimes even contradictory results in human studies need to be further investigated before this can be taken into account in clinical practice.

In summary, we conclude that, with the caveats indicated above, detection of platelet autoantibodies is truly a powerful diagnostic tool in the workup of patients suspected for ITP. In this respect, we agree with Vrbensky et al that the available GP-specific assays can at least be

used as a "rule in" test for ITP. We also conclude that we can and must further improve platelet autoantibody testing assays. For comparison of test accuracy in terms of sensitivity and specificity between laboratories, further standardization is necessary. In this regard, next to the GP tested, key parameters, like patient platelet counts and test cutoff levels, platelet numbers used for solubilization, GP-specific moab, and patient characteristics (routine laboratory requests or clinical cohorts of patients with suspected ITP; adults or children), should be standardized and reported. In addition to diagnosing ITP, a reliable GP-specific platelet autoantibody detection method can be used to further investigate the effects of the antibodies which will contribute to a more individualized treatment.

Declaration of Competing Interest

The authors declare that there is no conflict of interest.

References

[1] Cines DB, Bussel JB, Liebman HA, Luning Prak ET. The ITP syndrome: pathogenic and clinical diversity. Blood. 2009;113:6511–21.
[2] Zufferey A, Kapur R, Semple JW. Pathogenesis and therapeutic mechanisms in immune thrombocytopenia (ITP). J Clin Med. 2017;6.
[3] Kelton JG, Vrbensky JR, Arnold DM. How do we diagnose immune thrombocytopenia in 2018? Hematology Am Soc Hematol Educ Program. 2018;2018:561–7.
[4] Vrbensky JR, Moore JE, Arnold DM, Smith JW, Kelton JG, Nazy I. The sensitivity and specificity of platelet autoantibody testing in immune thrombocytopenia: a systematic review and meta-analysis of a diagnostic test. J Thromb Haemost. 2019;17:787–94.
[5] Provan D, Arnold DM, Bussel JB, Chong BH, Cooper N, Gernsheimer T, et al. Updated international consensus report on the investigation and management of primary immune thrombocytopenia. Blood Adv. 2019;3:3780–817.
[6] Arnold DM, Nazy I, Clare R, Jaffer AM, Aubie B, Li N, et al. Misdiagnosis of primary immune thrombocytopenia and frequency of bleeding: lessons from the McMaster ITP Registry. Blood Adv. 2017;1:2414–20.
[7] Porcelijn L, Huiskes E, Oldert G, Schipperus M, Zwaginga JJ, de Haas M. Detection of platelet autoantibodies to identify immune thrombocytopenia: state of the art. Br J Haematol. 2018;182:423–6.
[8] Bryant N, Watts R. Thrombocytopenic syndromes masquerading as childhood immune thrombocytopenic purpura. Clin Pediatr (Phila). 2011;50:225–30.
[9] Downes K, Megy K, Duarte D, Vries M, Gebhart J, Hofer S, et al. Diagnostic high-throughput sequencing of 2396 patients with bleeding, thrombotic, and platelet disorders. Blood. 2019;134:2082–91.
[10] Cines DB, Cuker A, Semple JW. Pathogenesis of immune thrombocytopenia. Presse Med. 2014;43:e49–59.
[11] Zhao C, Li X, Zhang F, Wang L, Peng J, Hou M. Increased cytotoxic T-lymphocyte-mediated cytotoxicity predominant in patients with idiopathic thrombocytopenic purpura without platelet autoantibodies. Haematologica. 2008;93:1428–30.
[12] Olsson B, Andersson PO, Jernas M, Jacobsson S, Carlsson B, Carlsson LM, et al. T-cell-mediated cytotoxicity toward platelets in chronic idiopathic thrombocytopenic purpura. Nat Med. 2003;9:1123–4.
[13] Chow L, Aslam R, Speck ER, Kim M, Cridland N, Webster ML, et al. A murine model of severe immune thrombocytopenia is induced by antibody- and CD8+ T cell-mediated responses that are differentially sensitive to therapy. Blood. 2010;115:1247–53.
[14] Kelton JG, Powers PJ, Carter CJ. A prospective study of the usefulness of the measurement of platelet-associated IgG for the diagnosis of idiopathic thrombocytopenic purpura. Blood. 1982;60:1050–3.
[15] Mueller-Eckhardt C, Kayser W, Mersch-Baumert K, Mueller-Eckhardt G, Breidenbach M, Kugel HG, et al. The clinical significance of platelet-associated IgG: a study on 298 patients with various disorders. Br J Haematol. 1980;46:123–31.
[16] Kelton JG, Murphy WG, Lucarelli A, Garvey-Williams J, Santos A, Meyer R, et al. A prospective comparison of four techniques for measuring platelet-associated IgG. Br J Haematol. 1989;71:97–105.
[17] Porcelijn L, Folman CC, Bossers B, Huiskes E, Overbeeke MA, C.E. v d Schoot, et al. The diagnostic value of thrombopoietin level measurements in thrombocytopenia. Thromb Haemost. 1998;79:1101–5.
[18] von dem Borne AE, Verheugt FW, Oosterhof F, von Riesz E, de la Riviere AB, Engelfriet CP. A simple immunofluorescence test for the detection of platelet antibodies. Br J Haematol. 1978;39:195–207.
[19] Hagenstrom H, Schlenke P, Hennig H, Kirchner H, Kluter H. Quantification of platelet-associated IgG for differential diagnosis of patients with thrombocytopenia. Thromb Haemost. 2000;84:779–83.
[20] Helmerhorst FM, Smeenk RJ, Hack CE, Engelfriet CP, von dem Borne AE. Interference of IgG, IgG aggregates and immune complexes in tests for platelet autoantibodies. Br J Haematol. 1983;55:533–45.
[21] van Leeuwen EF, van der Ven JT, Engelfriet CP, von dem Borne AE. Specificity of autoantibodies in autoimmune thrombocytopenia. Blood. 1982;59:23–6.
[22] Tanford C, Reynolds JA. Characterization of membrane proteins in detergent solutions. Biochim Biophys Acta. 1976;457:133–70.
[23] Bjerrum OJ. Immunochemical investigation of membrane proteins. A methodological survey with emphasis placed on immunoprecipitation in gels. Biochim Biophys Acta. 1977;472:135–95.
[24] Hagen I, Bjerrum OJ, Solum NO. Characterization of human platelet proteins solubilized with Triton X-100 and examined by crossed immunoelectrophoresis. Reference patterns of extracts from whole platelets and isolated membranes. Eur J Biochem. 1979;99:9–22.
[25] Porcelijn L, Huiskes E, de Haas M. Progress and development of platelet antibody detection. Transfus Apher Sci. 2020;59:102705.
[26] Woods Jr VL, Kurata Y, Montgomery RR, Tani P, Mason D, Oh EH, et al. Autoantibodies against platelet glycoprotein Ib in patients with chronic immune thrombocytopenic purpura. Blood. 1984;64:156–60.
[27] Woods Jr VL, Oh EH, Mason D, McMillan R. Autoantibodies against the platelet glycoprotein IIb/IIIa complex in patients with chronic ITP. Blood. 1984;63:368–75.
[28] McMillan R, Tani P, Millard F, Berchtold P, Renshaw L, Woods Jr VL. Platelet-associated and plasma anti-glycoprotein autoantibodies in chronic ITP. Blood. 1987;70:1040–5.
[29] Kiefel V, Santoso S, Weisheit M, Mueller-Eckhardt C. Monoclonal antibody–specific immobilization of platelet antigens (MAIPA): a new tool for the identification of platelet-reactive antibodies. Blood. 1987;70:1722–6.
[30] Furihata K, Nugent DJ, Bissonette A, Aster RH, Kunicki TJ. On the association of the platelet-specific alloantigen, Pena, with glycoprotein IIIa. Evidence for heterogeneity of glycoprotein IIIa. J Clin Invest. 1987;80:1624–30.
[31] Kiefel V, Santoso S, Kaufmann E, Mueller-Eckhardt C. Autoantibodies against platelet glycoprotein Ib/IX: a frequent finding in autoimmune thrombocytopenic purpura. Br J Haematol. 1991;79:256–62.
[32] He R, Reid DM, Jones CE, Shulman NR. Spectrum of Ig classes, specificities, and titers of serum antiglycoproteins in chronic idiopathic thrombocytopenic purpura. Blood. 1994;83:1024–32.
[33] Al-Samkari H, Rosovsky RP, Karp Leaf RS, Smith DB, Goodarzi K, Fogerty AE, et al. A modern reassessment of glycoprotein-specific direct platelet autoantibody testing in immune thrombocytopenia. Blood Adv. 2020;4:9–18.
[34] Beardsley D. Characterisation of platelet autoantigens (abstract). London: XX Congress ISBT; 1988 S-T-6-2.
[35] Meenaghan M, Judson PA, Yousaf K, Lewis L, Pamphilon DH. Antibodies to platelet glycoprotein V in polytransfused patients with haematological disease. Vox Sang. 1993;64:167–70.
[36] Joutsi L, Kekomaki R. Comparison of the direct platelet immunofluorescence test (direct PIFT) with a modified direct monoclonal antibody-specific immobilization of platelet antigens (direct MAIPA) in detection of platelet-associated IgG. Br J Haematol. 1997;96:204–9.
[37] Joutsi-Korhonen L, Javela K, Hormila P, Kekomaki R. Glycoprotein V-specific platelet-associated antibodies in thrombocytopenic patients. Clin Lab Haematol. 2001;23:307–12.
[38] Vollenberg R, Jouni R, Norris PAA, Burg-Roderfeld M, Cooper N, Rummel MJ, et al. Glycoprotein V is a relevant immune target in patients with immune thrombocytopenia. Haematologica. 2019;104:1237–43.
[39] Niessner H, Clemetson KJ, Panzer S, Mueller-Eckhardt C, Santoso S, Bettelheim P. Acquired thrombasthenia due to GPIIb/IIIa-specific platelet autoantibodies. Blood. 1986;68:571–6.
[40] Devine DV, Currie MS, Rosse WF, Greenberg CS. Pseudo-Bernard-Soulier syndrome: thrombocytopenia caused by autoantibody to platelet glycoprotein Ib. Blood. 1987;70:428–31.
[41] Deckmyn H, Chew SL, Vermylen J. Lack of platelet response to collagen associated with an autoantibody against glycoprotein Ia: a novel cause of acquired qualitative platelet dysfunction. Thromb Haemost. 1990;64:74–9.
[42] McMillan R, Bowditch RD, Tani P, Anderson H, Goodnight S. A non-thrombocytopenic bleeding disorder due to an IgG4-kappa anti-GPIIb/IIIa autoantibody. Br J Haematol. 1996;95:747–9.
[43] Porcelijn L, Huiskes E, Maatman R, de Kreuk A, de Haas M. Acquired Glanzmann's thrombasthenia caused by glycoprotein IIb/IIIa autoantibodies of the immunoglobulin G1 (IgG1), IgG2 or IgG4 subclass: a study in six cases. Vox Sang. 2008;95:324–30.
[44] De Cuyper IM, Meinders M, van de Vijver E, de Korte D, Porcelijn L, de Haas M, et al. A novel flow cytometry-based platelet aggregation assay. Blood. 2013;121:e70–80.
[45] van Bladel ER, Laarhoven AG, van der Heijden LB, Heitink-Polle KM, Porcelijn L, van der Schoot CE, et al. Functional platelet defects in children with severe chronic ITP as tested with 2 novel assays applicable for low platelet counts. Blood. 2014;123:1556–63.
[46] Kaushansky K. Thrombopoietin. N Engl J Med. 1998;339:746–54.
[47] Hitchcock IS, Kaushansky K. Thrombopoietin from beginning to end. Br J Haematol. 2014;165:259–68.
[48] Porcelijn L, Folman CC, de Haas M, Kanhai HH, Murphy MF, von dem Borne AE, et al. Fetal and neonatal thrombopoietin levels in alloimmune thrombocytopenia. Pediatr Res. 2002;52:105–8.
[49] Porcelijn L, Huiskes E, Onderwater-Van Den Hoogen L, Folman CC, Zwaginga JJ, De Haas M. Plasma thrombopoietin levels as additional tool in clinical management of thrombocytopenic neonates. Platelets. 2020;31:62–7.
[50] Peng J, Ma SH, Liu J, Hou Y, Liu XM, Niu T, et al. Association of autoantibody specificity and response to intravenous immunoglobulin G therapy in immune thrombocytopenia: a multicenter cohort study. J Thromb Haemost. 2014;12:497–504.
[51] Webster ML, Sayeh E, Crow M, Chen P, Nieswandt B, Freedman J, et al. Relative efficacy of intravenous immunoglobulin G in ameliorating thrombocytopenia induced by antiplatelet GPIIbIIIa versus GPIbalpha antibodies. Blood. 2006;108:943–6.

[52] Rumjantseva V, Hoffmeister KM. Novel and unexpected clearance mechanisms for cold platelets. Transfus Apher Sci. 2010;42:63–70.
[53] Hoffmeister KM. The role of lectins and glycans in platelet clearance. J Thromb Haemost. 2011;9(Suppl. 1):35–43.
[54] Li J, van der Wal DE, Zhu G, Xu M, Yougbare I, Ma L, et al. Desialylation is a mechanism of Fc-independent platelet clearance and a therapeutic target in immune thrombocytopenia. Nat Commun. 2015;6:7737.
[55] Quach ME, Dragovich MA, Chen W, Syed AK, Cao W, Liang X, et al. Fc-independent immune thrombocytopenia via mechanomolecular signaling in platelets. Blood. 2018;131:787–96.
[56] Rogier T, Samson M, Mourey G, Falvo N, Magy-Bertrand N, Ouandji S, et al. Antiplatelet antibodies do not predict the response to intravenous immunoglobulins during immune thrombocytopenia. J Clin Med. 2020;9. https://doi.org/10.3390/jcm9061998.
[57] Xu M, Li J, Neves MAD, Zhu G, Carrim N, Yu R, et al. GPIbalpha is required for platelet-mediated hepatic thrombopoietin generation. Blood. 2018;132:622–34.
[58] Porcelijn L, Schmidt DE, van der Schoot CE, Vidarsson G, de Haas M, Kapur R. Anti-glycoprotein Ibalpha autoantibodies do not impair circulating thrombopoietin levels in immune thrombocytopenia patients. Haematologica. 2020;105:e172–4.
[59] Gernsheimer TB. Thrombocytopenia in pregnancy: is this immune thrombocytopenia or...? Hematology Am Soc Hematol Educ Program. 2012;2012:198–202.
[60] Brighton TA, Evans S, Castaldi PA, Chesterman CN, Chong BH. Prospective evaluation of the clinical usefulness of an antigen-specific assay (MAIPA) in idiopathic thrombocytopenic purpura and other immune thrombocytopenias. Blood. 1996;88:194–201.
[61] Warner MN, Moore JC, Warkentin TE, Santos AV, Kelton JG. A prospective study of protein-specific assays used to investigate idiopathic thrombocytopenic purpura. Br J Haematol. 1999;104:442–7.
[62] He R, Reid DM, Jones CE, Shulman NR. Extracellular epitopes of platelet glycoprotein Ib alpha reactive with serum antibodies from patients with chronic idiopathic thrombocytopenic purpura. Blood. 1995;86:3789–96.
[63] Fujisawa K, Tani P, McMillan R. Platelet-associated antibody to glycoprotein IIb/IIIa from chronic immune thrombocytopenic purpura patients often binds to divalent cation-dependent antigens. Blood. 1993;81:1284–9.
[64] Kosugi S, Tomiyama Y, Shiraga M, Kashiwagi H, Mizutani H, Kanakura Y, et al. Platelet-associated anti-glycoprotein (GP) IIb-IIIa autoantibodies in chronic immune thrombocytopenic purpura mainly recognize cation-dependent conformations: comparison with the epitopes of serum autoantibodies. Thromb Haemost. 1996;75:339–45.
[65] Kekomaki R, Dawson B, McFarland J, Kunicki TJ. Localization of human platelet autoantigens to the cysteine-rich region of glycoprotein IIIa. J Clin Invest. 1991;88:847–54.
[66] Varon D, Karpatkin S. A monoclonal anti-platelet antibody with decreased reactivity for autoimmune thrombocytopenic platelets. Proc Natl Acad Sci U S A. 1983;80:6992–5.
[67] McMillan R, Lopez-Dee J, Loftus JC. Autoantibodies to alpha(IIb)beta(3) in patients with chronic immune thrombocytopenic purpura bind primarily to epitopes on alpha(IIb). Blood. 2001;97:2171–2.
[68] McMillan R, Wang L, Lopez-Dee J, Jiu S, Loftus JC. Many alphaIIbbeta3 autoepitopes in chronic immune thrombocytopenic purpura are localized to alphaIIb between amino acids L1 and Q459. Br J Haematol. 2002;118:1132–6.
[69] Kiyomizu K, Kashiwagi H, Nakazawa T, Tadokoro S, Honda S, Kanakura Y, et al. Recognition of highly restricted regions in the beta-propeller domain of alphaIIb by platelet-associated anti-alphaIIbbeta3 autoantibodies in primary immune thrombocytopenia. Blood. 2012;120:1499–509.
[70] Hou M, Stockelberg D, Kutti J, Wadenvik H. Glycoprotein IIb/IIIa autoantigenic repertoire in chronic idiopathic thrombocytopenic purpura. Br J Haematol. 1995;91:971–5.
[71] Escher R, Muller D, Vogel M, Miescher S, Stadler BM, Berchtold P. Recombinant human natural autoantibodies against GPIIb/IIIa inhibit binding of autoantibodies from patients with AITP. Br J Haematol. 1998;102:820–8.
[72] McMillan R. The pathogenesis of chronic immune thrombocytopenic purpura. Semin Hematol. 2007;44:S3–S11.
[73] Roark JH, Bussel JB, Cines DB, Siegel DL. Genetic analysis of autoantibodies in idiopathic thrombocytopenic purpura reveals evidence of clonal expansion and somatic mutation. Blood. 2002;100:1388–98.
[74] Stockelberg D, Hou M, Jacobsson S, Kutti J, Wadenvik H. Light chain-restricted autoantibodies in chronic idiopathic thrombocytopenic purpura, but no evidence for circulating clone B-lymphocytes. Ann Hematol. 1996;72:29–34.
[75] Arnold DM, Santoso S, Greinacher A. Platelet Immunology Scientific Subcommittee of the. Recommendations for the implementation of platelet autoantibody testing in clinical trials of immune thrombocytopenia. J Thromb Haemost. 2012;10:695–7.
[76] Campbell K, Rishi K, Howkins G, Gilby D, Mushens R, Ghevaert C, et al. A modified rapid monoclonal antibody-specific immobilization of platelet antigen assay for the detection of human platelet antigen (HPA) antibodies: a multicentre evaluation. Vox Sang. 2007;93:289–97.
[77] Vainchenker W, Deschamps JF, Bastin JM, Guichard J, Titeux M, Breton-Gorius J, et al. Two monoclonal antiplatelet antibodies as markers of human megakaryocyte maturation: immunofluorescent staining and platelet peroxidase detection in megakaryocyte colonies and in in vivo cells from normal and leukemic patients. Blood. 1982;59:514–21.
[78] McMillan R, Wang L, Tomer A, Nichol J, Pistillo J. Suppression of in vitro megakaryocyte production by antiplatelet autoantibodies from adult patients with chronic ITP. Blood. 2004;103:1364–9.
[79] Chang M, Nakagawa PA, Williams SA, Schwartz MR, Imfeld KL, Buzby JS, et al. Immune thrombocytopenic purpura (ITP) plasma and purified ITP monoclonal autoantibodies inhibit megakaryocytopoiesis in vitro. Blood. 2003;102:887–95.
[80] Houwerzijl EJ, Blom NR, van der Want JJ, Vellenga E, de Wolf JT. Megakaryocytic dysfunction in myelodysplastic syndromes and idiopathic thrombocytopenic purpura is in part due to different forms of cell death. Leukemia. 2006;20:1937–42.
[81] Grodzielski M, Di Buduo CA, Goette NP, Lev PR, Soprano PM, Heller PG, et al. Autoantibodies in immune thrombocytopenia affect the physiological interaction between megakaryocytes and bone marrow extracellular matrix proteins. Br J Haematol. 2018;183:319–23.
[82] Lev PR, Grodzielski M, Goette NP, Glembotsky AC, Espasandin YR, Pierdominici MS, et al. Impaired proplatelet formation in immune thrombocytopenia: a novel mechanism contributing to decreased platelet count. Br J Haematol. 2014;165:854–64.
[83] Shrestha S, Nazy I, Smith JW, Kelton JG, Arnold DM. Platelet autoantibodies in the bone marrow of patients with immune thrombocytopenia. Blood Adv. 2020;4:2962–6.
[84] Schmidt DE, Lakerveld AJ, Heitink-Polle KMJ, Bruin MCA, Vidarsson G, Porcelijn L, et al. Anti-platelet antibody immunoassays in childhood immune thrombocytopenia: a systematic review. Vox Sang. 2020;115:323–33.
[85] Hou M, Stockelberg D, Kutti J, Wadenvik H. Antibodies against platelet GPIb/IX, GPIIb/IIIa, and other platelet antigens in chronic idiopathic thrombocytopenic purpura. Eur J Haematol. 1995;55:307–14.
[86] Crossley A, Calvert JE, Taylor PR, Dickinson AM. A comparison of monoclonal antibody immobilization of platelet antigen (MAIPA) and immunobead methods for detection of GPIIb/IIIa antiplatelet antibodies in immune thrombocytopenic purpura. Transfus Med. 1997;7:127–34.
[87] Davoren A, Bussel J, Curtis BR, Moghaddam M, Aster RH, McFarland JG. Prospective evaluation of a new platelet glycoprotein (GP)-specific assay (PakAuto) in the diagnosis of autoimmune thrombocytopenia (AITP). Am J Hematol. 2005;78:193–7.
[88] Stockelberg D, Hou M, Jacobsson S, Kutti J, Wadenvik H. Detection of platelet antibodies in chronic idiopathic thrombocytopenic purpura (ITP). A comparative study using flow cytometry, a whole platelet ELISA, and an antigen capture ELISA. Eur J Haematol. 1996;56:72–7.
[89] Gaiger A, Neumeister A, Heinzl H, Pabinger I, Panzer S. HLA class-I and -II antigens in chronic idiopathic autoimmune thrombocytopenia. Ann Hematol. 1994;68:299–302.
[90] Panzer S, Hocker L, Rieger M, Vormittag R, Koren D, Dunkler D, et al. Agonist-inducible platelet activation in chronic idiopathic autoimmune thrombocytopenia. Eur J Haematol. 2007;79:198–204.
[91] Najaoui A, Bakchoul T, Stoy J, Bein G, Rummel MJ, Santoso S, et al. Autoantibody-mediated complement activation on platelets is a common finding in patients with immune thrombocytopenic purpura (ITP). Eur J Haematol. 2012;88:167–74.
[92] Fabris F, Casonato A, Randi ML, Luzzatto G, Girolami A. Clinical significance of surface and internal pools of platelet-associated immunoglobulins in immune thrombocytopenia. Scand J Haematol. 1986;37:215–20.
[93] Fabris F, Casonato A, Zanchetta R, Cortellazzo S, Busolo F, Girolami A. A microplate enzyme-linked immunospecific assay (ELISA) detecting unbound anti-platelet antibodies. Folia Haematol Int Mag Klin Morphol Blutforsch. 1984;111:66–71.
[94] Meyer O, Agaylan A, Borchert HH, Aslan T, Bombard S, Kiesewetter H, et al. A simple and practical assay for the antigen-specific detection of platelet antibodies. Transfusion. 2006;46:1226–31.
[95] Nurden P, Nurden AT. Is the mysterious platelet receptor GPV an unsuspected major target for platelet autoantibodies? Haematologica. 2019;104:1103–5.
[96] Porcelijn L, Huiskes E, Oldert G, Fijnheer R, Schipperus MR, Zwaginga JJ, et al. Detection of platelet autoantibodies revisited to identify immune thrombocytopenia. Blood. 2015;126:1142.
[97] Porcelijn L, Huiskes E, Schipperus M, van der Holt B, de Haas M, Zwaginga JJ, et al. Lack of detectable platelet autoantibodies is correlated with nonresponsiveness to rituximab treatment in ITP patients. Blood. 2017;129:3389–91.
[98] Kosugi S, Tomiyama Y, Honda S, Kashiwagi H, Shiraga M, Tadokoro S, et al. Anti-alphavbeta3 antibodies in chronic immune thrombocytopenic purpura. Thromb Haemost. 2001;85:36–41.
[99] Fabris F, Scandellari R, Randi ML, Carraro G, Luzzatto G, Girolami A. Attempt to improve the diagnosis of immune thrombocytopenia by combined use of two different platelet autoantibodies assays (PAIgG and MACE). Haematologica. 2002;87:1046–52.
[100] Chan H, Moore JC, Finch CN, Warkentin TE, Kelton JG. The IgG subclasses of platelet-associated autoantibodies directed against platelet glycoproteins IIb/IIIa in patients with idiopathic thrombocytopenic purpura. Br J Haematol. 2003;122:818–24.
[101] Fabris F, Scandellari R, Ruzzon E, Randi ML, Luzzatto G, Girolami A. Platelet-associated autoantibodies as detected by a solid-phase modified antigen capture ELISA test (MACE) are a useful prognostic factor in idiopathic thrombocytopenic purpura. Blood. 2004;103:4562–4.
[102] Tomer A, Koziol J, McMillan R. Autoimmune thrombocytopenia: flow cytometric determination of platelet-associated autoantibodies against platelet-specific receptors. J Thromb Haemost. 2005;3:74–8.
[103] Meyer O, Agaylan A, Bombard S, Kiesewetter H, Salama A. A novel antigen-specific capture assay for the detection of platelet antibodies and HPA-1a phenotyping. Vox Sang. 2006;91:324–30.
[104] He Y, Zhao YX, Zhu MQ, Wu Q, Ruan CG. Detection of autoantibodies against platelet glycoproteins in patients with immune thrombocytopenic purpura by flow cytometric immunobead array. Clin Chim Acta. 2013;415:176–80.