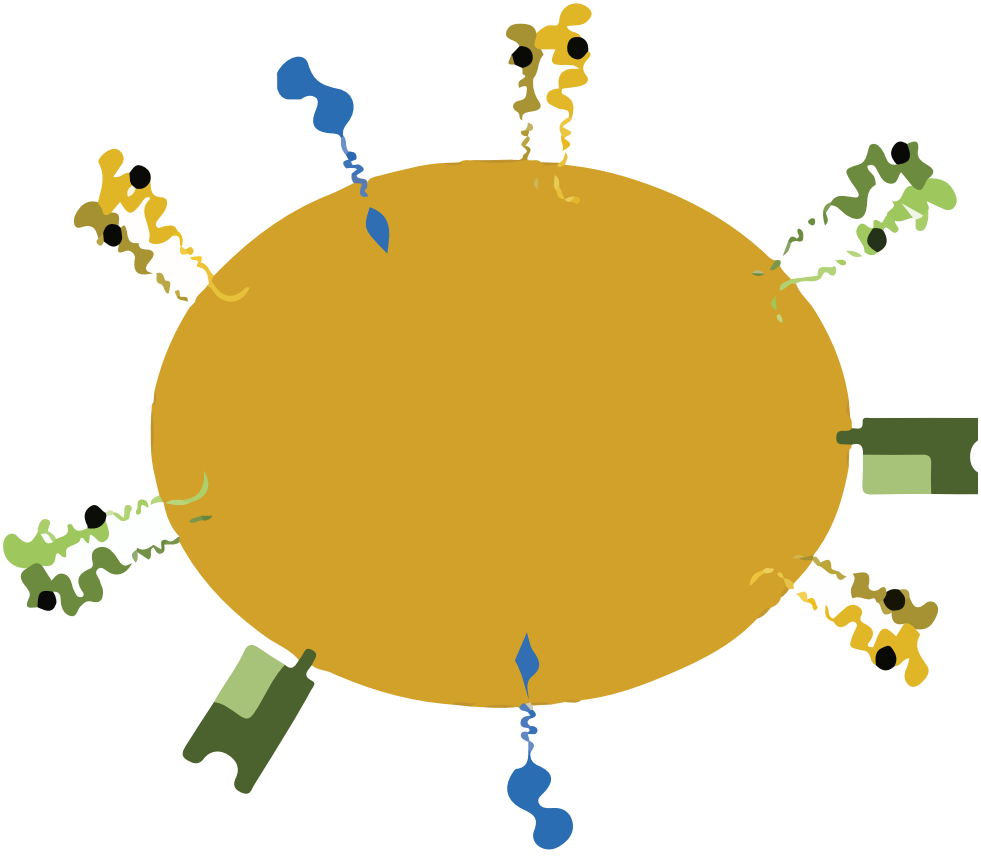
The diagnostic value of plasma thrombopoietin levels and platelet autoantibodies

Porcelijn, L.



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CHAPTER 8

Evolution and utility of antiplatelet autoantibody testing in patients with immune thrombocytopenia

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Evolution and utility of antiplatelet autoantibody testing in patients with immune thrombocytopenia

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Abbreviations

ACE	antigen capture ELISA
AMR	Aswell-Morell receptor
ASCA	antigen specific capture assay
ASPA	antigen specific particle assay
BM	bone marrow
DNA	deoxyribonucleic acid
FNAIT	fetal/neonatal alloimmune thrombocytopenia
GP	glycoprotein
hc	healthy control
HPA	Human platelet antigen
ISTH	International Society on Thrombosis and Haemostasis
ITP	immune thrombocytopenia
IVIg	intravenous immunoglobuline
LBD	lectine binding domain
MACE	modified antigen capture ELISA
MAIPA	monoclonal antibody immobilization of platelet antigens
MK	megakoryocyte
moab	monoclonal antibody
OD	optical density
PIFT	platelet immunofluorescence test
SD	standard deviation
Tpo	thrombopoietin

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 work-up 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 cut-off 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 seems 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

General introduction

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 over 40 years. More recent guidelines do indicate that performing glycoprotein 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 glycoprotein 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 work-up 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 value of a diagnostic gold-standard is proven in several studies. The McMaster ITP registry set up in Canada showed that 36 of 295 (12.2%) adult patients initially diagnosed as 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 (i.e. independent of the autoantibody detection results), ITP could be excluded for 76 of 165 (46%) patients.[7] Also the ten-year retrospective chart review by Bryant et al. of a large cohort (n=492) 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, due to 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 for whom 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' is 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 two-step assays measured platelet-bound and circulating platelet-reactive antibodies, making use of fluorescence-labeled anti-human-immunoglobulines.[14-16] The two-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 (i.e., 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, e.g. 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 non-specific results, solubilization of the platelet membrane and extraction of the membrane proteins, retaining their antigenicity, with non-ionic detergents was described at the time.[22-25] Together with the availability of GP-specific monoclonal antibodies (moab), this led to the development of glycoprotein-specific platelet antibody detection methods. After a first experimental approach by Woods et al (1984) with immobilization of glycoproteins IIb/IIIa and Ib/IX on microtiterplates, two more sensitive methods were introduced, i.e. 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 microtiterplate wells coated with a GPIIb/IIIa specific moab, enabling them to confirm the presence of GPIIb/IIIa specific autoantibodies in plasma from five 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 three of 106 plasma's 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, while 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 two of 34 samples responded with both GP, which later turned out to be too low a percentage. The development of the antigen-capture ELISA (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%) GPIIb/IIIa specific, 19 (33%) GPIb/IX specific and 22 (38%) reactive with both GP.[31]

An overview of studies using GP-specific methods for the detection of autoantibodies is shown in Table1. In most studies, approximately 60-80% of autoantibodies react with GPIIb/IIIa and 50% with GPIb/IX. The majority of samples contain antibodies with both types of GP specificities, but still a significant percentage (10-40%) reacts with only one 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 immune thrombocytopenia

Author	assay	material tested	number of pl used in test* x 10^6	patients	Healthy controls	non-ITP	Glycoprotein specific antibodies						pat pl counts	assay cut-off	GP specific MoAB
				pos/total (%)	pos/ total (%)	pos/ total (%)	GPIIb/IIIa	GPIb/IX	GPIa/IIa	GPV	GPIV	combi GP			
Woods 1984a[27]	ELISA	plasma		5/56 (9%)	0/16	0/34	5	nt	nt	nt	nt	nt		mean controls + 3SD	2G12 (IIb/IIIa)
Woods 1984b[26]	ELISA	plasma		3/73 (4%)	0/22	0/59	nt	3	nt	nt	nt	nt		mean controls + 3SD	AP1 (Ib)
McMillan 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#	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 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 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 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 + 6SD	VIPL1 (IIb/IIIa) , VIPL3 (IIb/IIIa), FMC25 (IX), SZ1 (Ib/IX)
		sera		5/45 (11%)					nt	nt	nt		4-700	Mean controls + 6SD	
Hou 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 + 3SD	AP1 (Ib), AP2 (IIb/IIIa)
Stockelberg 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 + 3SD	AP1 (Ib), AP2 (IIb/IIIa)
Brighton 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 + 3SD	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 + 3SD	SZ21 (IIIa), SZ22 (IIb), AP2 (IIb/IIIa, AK2 (Ibα), FMC25 (IX)
Crossley 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 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 1998[17]	MAIPA	platelets	15 (IIb/IIIa) 40 (other GP)	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)
Warner 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 2001[98]	MACE	eluates	50	18/47 (38%)			18/18 (100%)	nt	nt	nt	nt	nt		mean controls + 3SD	AP3 (IIIa)
Fabris 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 + 3SD	AP2 (IIb/IIIa), 142.1 (Ib/IX), 143.1 (Ia/IIa)
Chan 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 2004[101]	commercial MACE	platelets		25/50 (50%)			18/25 (72%)	15/25 (60%)	2/25 (8%)			8/25 (32%)	< 100		
Davoren 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 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 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 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 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 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 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 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%)	<100	0.13	P58 (IV)
Vollenberg 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%)		0.2	Gi5 (IIb/IIIa, FMC25 (IX), SW16 (V)

Al-Samkari 2020[33]	commercial ELISA	eluates		205/228 (90%)		24/108 (22%)	269/280* (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		

**Number of platelets used in test: the number of platelets used per GP-specificity, to test the binding of GP-specific autoantibodies*
 # only: meaning only reactive with this GP, not with the other tested GP
Empty cells: no data are available in publication
nt: not tested

2. Other GP 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 three (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 ELISA (PAKAuto) confirmed the almost non-occurrence of autoantibody binding exclusively to GPIa/IIa or GPIV.

Glycoprotein V as a target for autoantibodies was first reported by Beardsley (1988) in a case of childhood ITP.[34] In 1993, Meenaghan showed that the majority of GP reactive antibodies in multi-transfused patients with bone marrow 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%, six specific and six 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 a higher average OD values for GPV compared with GPIb/IX and GPIIb/IIIa (Figure 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 glycoprotein 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 GP. First case reports of, so called, acquired Glanzmann disorder and acquired Bernard Soulier syndrome were already published in 1987 by Niessner et al., respectively Devine et al.[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. Also a case of severe impaired response of platelets to collagen, due to GPIa/IIa specific autoantibodies blocking the collagen receptor, has 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, both in adult and in 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.

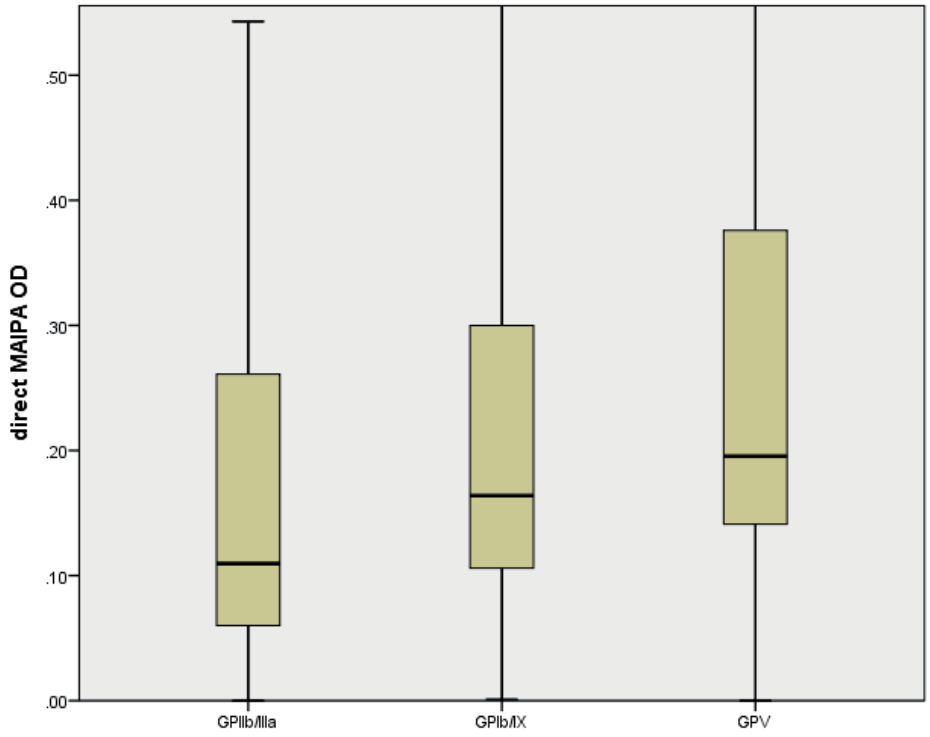

Figure 1: Routine autoantibody detection with positive results (i.e. at least one of the tested GP shows positive results) in direct MAIPA (n=754)
For 754 positive direct MAIPA results (i.e. at least one of the glycoproteins IIb/IIIa, Ib/IX or V shows OD values > 0.130) the results for the different 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 x 106 platelets for GPIIb/IIIa and 40 x 106 platelets for GPIb/IX and GPV. The effect of raising the platelet number for GPIIb/IIIa is shown in Figure 4.

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 immune thrombocytopenia (ITP) and FNAIT (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 de-sialylation 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 de-sialylation 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 Al-Samkari et al. and Rogier et al. studies, 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 < 100 instead of < 150 x 109/L

For several reasons, the platelet count of the patient is important in the detection of autoantibodies. First, for the categorization of patients in the group of patients with suspected ITP. For instance, in our laboratory we frequently receive autoantibody requests for pregnant women with platelet counts between 100 and 150 x 109/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 already, Brighton showed a non-statistically 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 glycoprotein specific antigen capture

assay was negative for ITP patients with platelet counts > 100 x 109/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 four platelet count groups, detection of autoantibodies became less sensitive for patients with platelet counts above 100 x 109/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 x 10 x 109/L.

6. The impact of the glycoprotein specific monoclonal antibodies used in the assay on the test results

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

Kiefel et al (1991) showed for three ITP patients with auto-antibodies 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 six 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 six 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; Figure 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 Figure 3. For the majority of 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 and He.[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, e.g. 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. Already in 1983, Varon and Karpatkin 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 (CHO) 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, 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 two ITP patients and Fab fragments from two human monoclonal anti-GPIIb/IIIa, both inhibiting the binding of anti-GPIIb/IIIa from other ITP patients (Hou 1995, Escher 1998, McMillan 2007).[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 ISTH recommended to use moab to each of the GPIIb (e.g. SZ22) and GPIIIa (e.g. SZ21) subunits or to the intact GPIIb/IIIa (e.g. Gi5, AP2, Raj-1); GPIb/IX (e.g. the GPIbα specific AP1 or the GPIX specific FMC25).[75] In our hands C17 (GPIIb/IIIa) SW16 (GPV), and FMC25 (GPIX) replacing MB45 (GPIbα) show best results in the MAIPA.

7. The impact of cut-off values in the assay

To differentiate specific signals from the noise, the assay cut-off value to be used is, of course, very dependent on the test specifics. E.g. 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 cut-off values to determine positive vs negative results vary among papers, 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 cut-off value to OD = 0.13 (mean 462 healthy controls + 3SD), without compromising specificity.[7] In a series of 754 routine request samples with at least one of the glycoproteins IIb/IIIa, Ib/IX or V reacting positive 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 Figure 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 cut-off value of the mean of healthy controls + 3SD is still too high to sensitively detect platelet autoantibodies. Illustrating in this context is the high sensitivity of 90% at the expense of specificity (78%) found by Al-Samkari 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 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 not only a disorder causing increased platelet destruction, but also decreased platelet production. Glycoproteins are already expressed on megakaryocytes (MK) during maturation[77], and GPIIb/IIIa, GPIb/IX and GPIa/IIa autoantibodies are known to cause inhibition of MK maturation, as well as pro-platelet and platelet formation.[78, 79] Although, most ITP patients show normal MK numbers in the bone marrow (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 –GPIb/IX interfered with the MK-fibrinogen, respectively -von Willebrand Factor interaction, leading to functional abnormalities and inhibited pro-platelet 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 out of ten patients with detectable antibodies in the BM could not be detected in the peripheral blood. All controls, i.e. 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%.

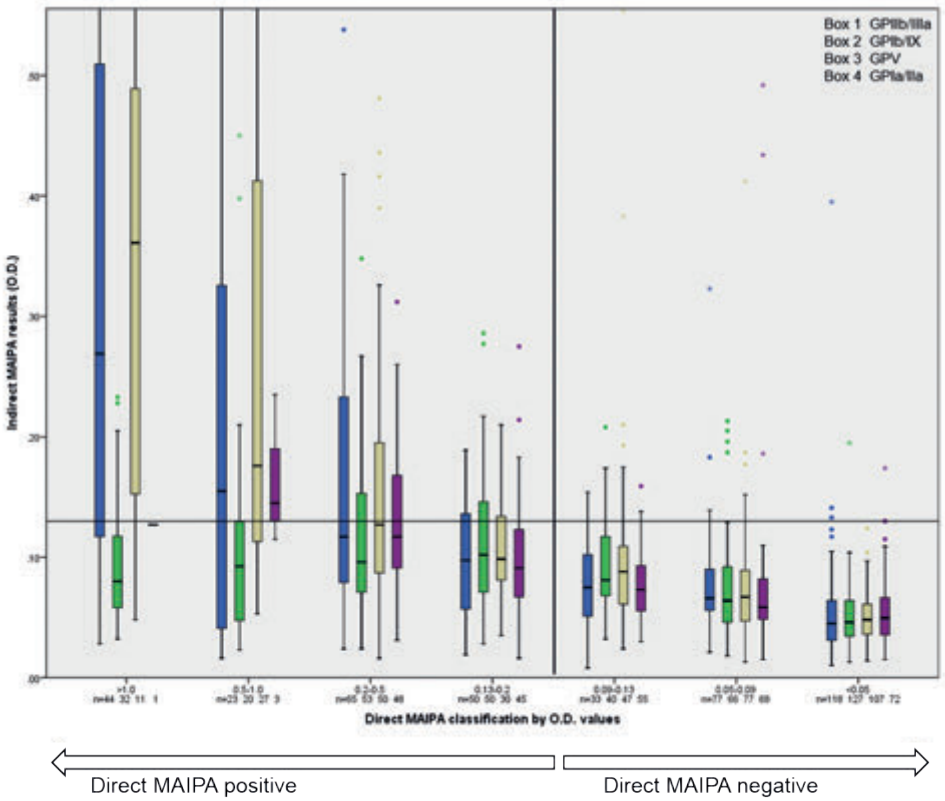

Figure 2: Indirect versus direct autoantibody detection with MAIPA (n=400)
Comparing direct (platelet-associated, groups categorized in OD ranges) and indirect (free-circulating) MAIPA results, shows a highly significant correlation. Interesting is the correlation continuing below the cut-off level of 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 Figure 3).

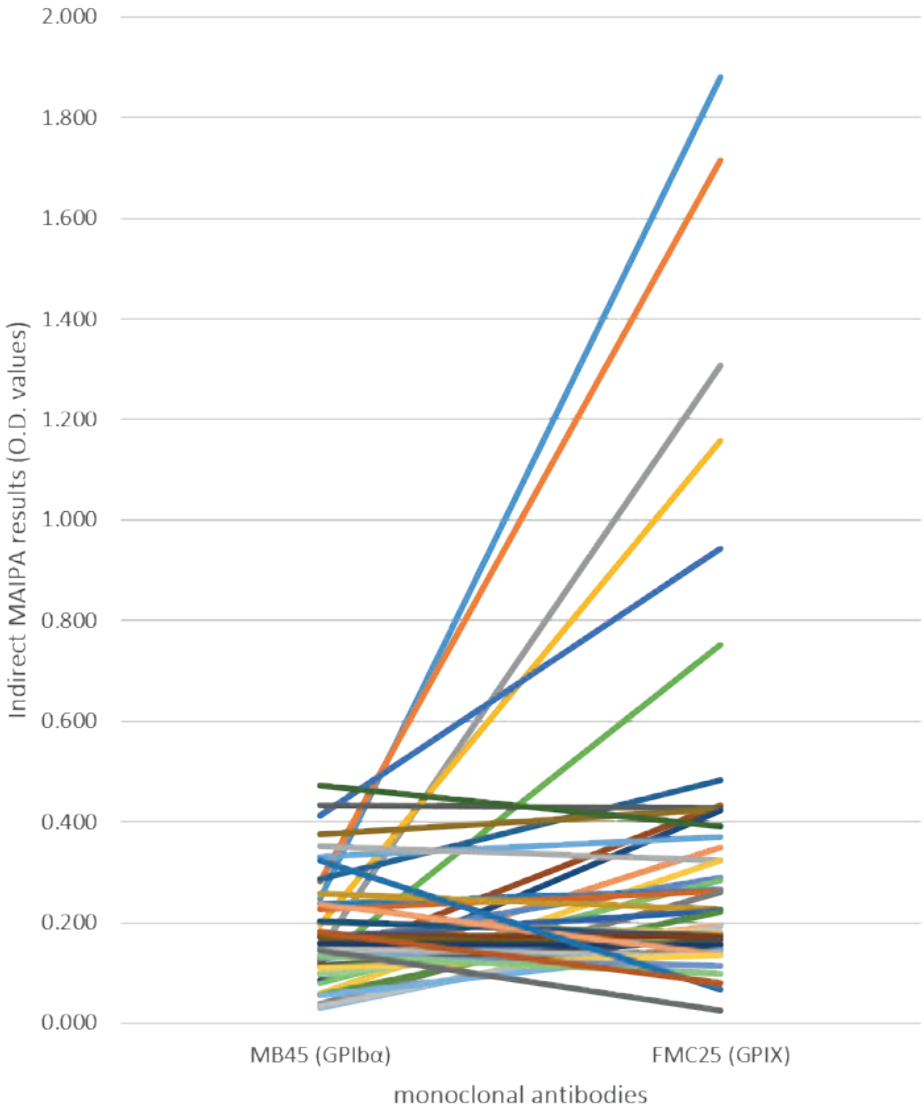

Figure 3: Comparison anti-GPIb/IX positive indirect MAIPA results if using the moab MB45 or FMC25 (n=45)
The indirect MAIPA results, using MB45 and FMC25 for 45 positive (i.e. positive with MB45 and/or positive with FMC25) are shown.

9. Discussion

We would like to emphasize that auto antibody 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 eighteen 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 can be useful for ruling in ITP, but a negative test 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 eighteen studies included in the paper of Vrbensky and completed the series with the four studies introducing GP-specific assays[26-28, 31] and nine studies also giving information on the GP specificity of platelet autoantibodies, including the recent papers by Vollenberg and Al-Samkaria.[17, 32, 36, 85-88] To understand the significant differences in sensitivity found in the studies, we compared some important aspects. First, five of nine eligible studies used by Vrbensky 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 one 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 is probably very much depending on the test characteristics. For instance, Vollenberg et al. did not see any differences in antibody load for the different GP[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 (Figure 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 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 GP. For optimal use of the limited patient platelets available for testing, we use 15 x 106 platelets in the MAIPA for GPIIb/IIIa, versus 40 x 106 for GPIb/IX and for GPV. The effect of increasing the input from 15 to 40 x 106 platelets per test is shown in Figure 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 x 106 per test. As shown in Table 1, the platelet numbers used for testing vary significantly between studies, which also complicates comparing the results. Second, patients with platelet counts > 100 x 109/L were included in three[60, 89, 90] of the nine studies and platelet counts were not mentioned in three other studies[61, 91, 94]. In our opinion, it is important to limit testing to the group of patients with platelet counts below 100 or better still below 80 x 109/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) + 3SD or 0.200 as cut-off value.[60, 61, 89, 94] The mean of healthy controls is, of course, dependent on the background signals and varies significantly between studies. Using hc + 3SD 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 one hour increment is often zero, indicating platelets are almost instantly opsonized and removed from circulation. One explanation could be

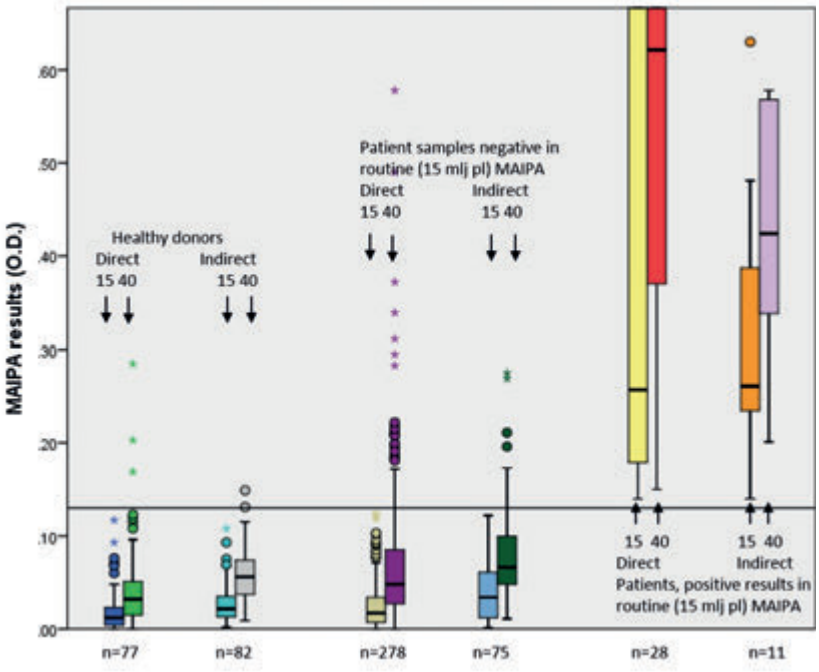

Figure 4: 15 or 40 x 106 platelets used in MAIPA for detection of GPIIb/IIIa reactive autoantibodies
The effect of increasing the number of platelet from 15 to 40 x 106 used in the direct and indirect MAIPA is shown.

that antibody production, platelet opsonization and removal occurs locally in the spleen and free-circulating autoantibodies in the peripheral blood are less detectable. Considering that, direct and indirect autoantibody test results (Figure 2) correlate even below our cut-off 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 (Figure 2). These different results might well be caused by the different assays used. In the PAKAuto GP are already isolated and bound to the microtiterplate wells, whereas in MAIPA intact platelets are used. The number of GP per well may vary significantly in PAKAuto, but are reasonably comparable between indirect and direct MAIPA, using the same number of platelets.

In addition to the detection of glycoprotein 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 glycoprotein 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 (i.e. platelet counts), serial testing can be supportive for treatment policy, especially for patients with more possible causes for thrombocytopenia. The benefits of knowing the glycoprotein 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 (e.g. splenectomy and thrombopoietin) on antibody production. Studying well categorized ITP patients, using reliable glycoprotein-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, thrombopoietin production, GPIbα de-sialylation 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 work-up 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 cut-off levels, platelet numbers used for solubilization, GP specific moab and patient characteristics (routine laboratory requests or clinical cohorts patients; adults or children) should be standardized and reported. In addition to diagnosing ITP, a reliable glycoprotein-specific platelet autoantibody detection method can be used to further investigate the effects of the antibodies which will contribute to a more individualized treatment.

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