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MINIREVIEWS

Role of novel oral anticoagulants in the management and prevention of venous thromboembolism

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Abstract

Venous thromboembolism (VTE) encompasses deep vein thrombosis and pulmonary embolism and is a major health burden, both medically and economically. Anticoagulation is the primary treatment and can be divided into three stages: initial, long term and extended treatment. Initial anticoagulation is given to reduce the risk of complications including fatal pulmonary embolism, while long term and extended treatment are aimed at prevention of recurrent VTE. Until recently, initial anticoagulation has only been achievable with administration of parental agents such as unfractionated or low molecular weight heparin, while vitamin K antagonists such as warfarin, have been the mainstay of long term and extended treatment. Factor-Xa inhibitors and direct thrombin inhibitors are oral anticoagulants that are being increasingly utilized as an alternative form of anticoagulation. This article aims to review the current guidelines in the management of VTE, the recent literature regarding novel anticoagulants in VTE, suggested treatment regimes and limitations.

Key words: Factor Xa inhibitors; Venous thromboembolism; Novel oral anticoagulants; Pulmonary embolism; Direct thrombin inhibitors

Core tip: Novel oral anticoagulants (NOACs) are emerging as viable alternatives to Vitamin K antagonist (VKA) in the treatment of venous thromboembolism. Trials have shown that they are as efficacious as current standard treatment with low-molecular-weight heparin followed by VKA, and have potentially less bleeding associated with them. The regimes are simple and no monitoring is required and therefore it has the potential to reduce the burden of anticoagulation. Caution is required however, as testing of anticoagulant effect is limited and patient selection is important as many of the NOACs are metabolized in the liver and cleared by the kidney.

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INTRODUCTION

Venous thromboembolism (VTE) is a common

condition[1] that may be lethal or lead to chronic disease and disability[2,3]. Warfarin has been the "gold standard" in oral anticoagulation for more than 50 years however its slow onset of action necessitates treatment with parenteral anticoagulation for at least 5 d. This means that hospital admission is often required for the immediate treatment phase while warfarin reaches therapeutic levels. The therapeutic dose of warfarin for individuals also varies, reflecting differences in dietary vitamin K consumption, drug interactions and genetic polymorphisms in enzymes of warfarin metabolism. As a result, patient education, compliance and frequent monitoring are essential to ensure anticoagulation remains in the narrow therapeutic window. Sub therapeutic levels can increase the risk of thrombosis while excessive anticoagulation can result in bleeding[4]. Also, while rare, warfarin skin necrosis can be a devastating complication of warfarin therapy[5].

The novel oral anticoagulants (NOACs) have a fast onset of action and more predictable anticoagulation effect. Some of the NOACs, rivaroxaban and apixaban, can be administered without the need for initial parenteral anticoagulation and they can all be given in fixed doses with no, or little need for monitoring of anticoagulant effect. This has the potential to reduce the burden of anticoagulation for the patient, physician and the healthcare system by introducing regimes that are easy to administer and monitor. Without the need for parental anticoagulation for some of the NOACs, there is potential for patients with low risk pulmonary embolisms to be discharged early and managed at home[6].

The decision regarding length of treatment is based on the benefits and harms of continuing anticoagulation as assessed by the physician, as well as on the patient's preference. There have been many clinical trials evaluating various durations of anticoagulant treatment for VTE[7-10]. These studies showed that anticoagulation should be continued for at least 3 mo, as treatment for shorter durations resulted in a significant increase in recurrent VTE. There was a similar rate of recurrent VTE after longer treatments of 6 to 12 mo. Studies have also shown that indefinite treatment reduces the risk of recurrent VTE by about 90% but there was also an increase in major bleeding by 1% or higher annually[11,12]. The risk of recurrent VTE was lowest in those with an isolated below knee DVT and was similar after proximal DVT and PE. It was also noted that recurrence was lower if the thrombosis was provoked by a temporary risk factor compared to an unprovoked thrombosis (HR = 0.55, 95%CI: 0.41-0.74)[13].

These results are reflected in existing guidelines in VTE management[6,14,15]. Anticoagulation for 3 mo is recommended for PE secondary to a transient, reversible risk factor. The guidelines regarding extended treatment however are less clear and promote extended treatment in patients with unprovoked PE that have low bleeding risk. They also recommend the risk-benefit ratio for anticoagulation be re-assessed at regular intervals.

With the advent of novel oral anticoagulants, the risk-benefit ratio has potentially shifted, with many of the new agents associated with lower bleeding complications than traditional treatment with warfarin. They also have simpler regimes that do not require frequent monitoring. We aim to review the current literature and examine the pharmacology of the novel anticoagulants as well as their efficacy and safety in the immediate, long term and extended treatment of VTE.

NOACs

Dabigatran

Dabigatran, an oral direct thrombin inhibitor, is the active form of the double prodrug, dabigatran etelixate. Once absorbed from the gastrointestinal tract, bioconversion of dabigatran occurs in the gut and is completed in the liver. Cytochrome P450 does not play any role in its metabolism and therefore the risk of drug interactions is low[16,17]. The bioavailability of dabigatran is however, only 8% and absorption from the gut is reliant on an acid environment, meaning that proton pump inhibitors can reduce drug absorption. Peak concentrations occur in about 2 h and the half life is 12-17 h. About 80% of dabigatran is excreted unchanged by the kidneys and therefore plasma concentrations can increase in renal impairment.

The pivotal studies looking at the role of dabigatran in VTE were the RE-COVER[18] and RECOVER Ⅱ studies[19]. RE-COVER was a randomized double blind, non-inferiority study of 2539 patients comparing dabigatran with warfarin. In this phase three study, 69% of patients had DVT, 21% had PE and 10% had both PE and DVT. Both the dabigatran and warfarin groups received a full 5 d course of low molecular weight heparin before starting the drug. The dabigatran dose was 150 mg twice daily and the target INR in the warfarin group was 2-3, with intended treatment length of 6 mo.

Recurrent VTE occurred in 2.4% in the dabigatran group and 2.1% in warfarin (HR = 1.10, 95%CI: 0.65-1.84), indicating that dabigatran was non-inferior to warfarin. There was no statistically significant difference in major bleeding with 1.6% in dabigatran compared with 1.9% in warfarin (HR = 0.82, 95%CI: 0.45-1.48) having major bleeding events. There was however a reduction in major or clinically significant bleeding in the dabigatran group (5.6%) compared with warfarin (8.8%) (HR = 0.63, 95%CI: 0.47-0.84).

RE-COVER Ⅱ had a similar design and tested an additional 2589 patients with acute VTE. The population was similar to the RE-COVER trial and had 68% of patients with DVT, 23% with PE and 9%

with both DVT and PE. Results were similar to the previous trial and showed that dabigatran was non-inferior to warfarin. In this trial, VTE occurred in 2.3% of the dabigatran group compared with 2.2% in warfarin group (HR = 1.08, 95%CI: 0.64-1.80). There was again no difference in major bleeding with 1.2% in dabigatran and 1.7% in warfarin (HR = 0.69, 95%CI: 0.36-1.32) but again there was a reduction in major or clinically relevant non-major bleeding with 5% in dabigatran compared with 7.9% in warfarin (HR = 0.62, 95%CI: 0.45-0.84).

Dabigatran in extended therapy was evaluated in RE-MEDY[20] and RE-SONATE[20]. RE-MEDY was a phase Ⅲ trial comparing extended therapy with dabigatran 150 mg twice daily with warfarin (INR 2-3). There were 2866 patients with VTE who had completed 3-12 mo of anticoagulation with either warfarin or dabigatran as part of the RE-COVER and RE-COVER Ⅱ trials enrolled. Dabigatran was non-inferior to warfarin with recurrent VTE occurring in 1.8% of patients treated with dabigatran compared with 1.3% of patient treated with warfarin (HR = 1.44, 95%CI: 0.78-2.64). There seemed to be an almost 50% reduction in major bleeding in the dabigatran group compared with warfarin; (0.9%) *vs* (1.8%) respectively, however the confidence interval was wide and was not statistically significant (HR = 0.52, 95%CI: 0.27-1.02). There was however, a significant reduction in major or clinically relevant bleeding with almost double the amount of bleeding in warfarin group (10.2%) compared with dabigatran (5.6%) (HR = 0.54, 95%CI: 0.41-0.71).

RE-SONATE was a phase Ⅲ study in 1353 patients that compared dabigatran 150 mg twice daily to placebo for 6 mo. All patients in this study had completed 6 to 18 mo of treatment for VTE with dabigatran or warfarin. Recurrent VTE was less frequent in the dabigatran group with an incidence of 0.4% *vs* 5.6% in the placebo arm (HR = 0.08, 95%CI: 0.02-0.25). This however, was at the expense of increased bleeding, with major bleeding occurring in 2 patients (0.3%) in dabigatran compared with none in placebo. Major or clinically significant bleeding was also more common in the dabigatran group (5.3%) compared with placebo (1.8%) (HR = 2.92, 95%CI: 1.52-5.60).

These results highlight that dabigatran, a direct thrombin inhibitor, are non-inferior to warfarin in the long term and extended treatment for VTE. There is also the added benefit of a reduction in clinically relevant and overall bleeding in dabigatran compared with warfarin. While dabigatran was effective at reducing recurrent VTE in extended therapy compared to placebo, this was, not surprisingly, associated with increased bleeding. Treatment with dabigatran however still necessitates immediate treatment with parenteral therapy, making treatment regimes with this drug more complex than some of the other novel anticoagulants. The predictable anticoagulation profile means that it does not need to be monitored and therefore would be less cumbersome than warfarin therapy. As the majority of dabigatran is cleared by the kidney, use of this drug is contra-indicated in severe renal impairment.

Rivaroxaban

Rivaroxaban is a direct, selective inhibitor of factor Ⅹa. It is well absorbed from the gut and has a bioavailability of greater than 80%[21]. Peak concentrations occur in about 3 h and its half life is 5-9 h in the young and 11-13 h in the elderly. It is metabolized in the liver *via* CYP3A4, CYP2C8 as well as CYP-independent mechanisms[22] and is therefore contraindicated in severe liver disease. It has few drug interactions, however drugs that have potent affects on CYP3A4 may alter plasma concentrations. Rivaroxaban is excreted *via* the kidney as unchanged drug (30%-40%) and as its metabolites (30%-40%), with the remainder excreted as unchanged drug in faeces. Intestinal excretion of rivaroxaban is partly mediated by P-glycoprotein, and potent inhibitors can increase drug concentrations. Because of its renal clearance, rivaroxaban should be used with caution in patients with renal impairment.

Unlike studies in other NOACs, the efficacy of rivaroxaban was assessed in DVT and PE separately in the EINSTEIN–DVT and EINSTEIN-PE studies.

EINSTEIN-DVT[23] was a phase Ⅲ, open label, event driven study comparing rivaroxaban (15 mg twice daily for 3 wk, followed by 20 mg once daily) with subcutaneous enoxaparin followed by a vitamin K antagonist for 3, 6 or 12 mo in patients with acute, symptomatic DVT. There were 3449 patients included in the study, most of whom received treatment for 6 mo (63%). Rivaroxaban was non-inferior to warfarin with recurrent VTE occurring in 2.1% of the rivaroxaban group and 3.0% in the warfarin group (HR = 0.68, 95%CI: 0.44-1.04). While there was a trend towards less major bleeding with rivaroxaban (0.8%) compared with warfarin (1.2%), this did not reach statistical significance (HR = 0.65; 95%CI: 0.33-1.30). There was also no significant difference in clinically relevant non major bleeding between the two groups (rivaroxaban 7.3% and warfarin 7.0%).

EINSTEIN-PE[24] had the same design in patients with acute PE, with or without DVT. There were 4832 patients enrolled, making this the largest cohort of patients with PE to be studied across the various phase Ⅲ NOAC studies. Most patients were treated for 6 mo (57.4%) with a large proportional treated for 12 mo (37.5%) and a smaller portion for 3 mo (5.2%). Rivaroxaban was non-inferior to warfarin, with recurrent VTE occurring in 2.1% in the rivaroxaban group and 1.8% in the warfarin group (HR = 1.12, 95%CI: 0.75-1.68). Major bleeding was halved in the rivaroxaban group (1.1%) compared with standard therapy (2.2%) (HR = 0.49; 95%CI:

0.31-0.79). There was however again, no significant difference in the major or clinically significant non major bleeding; 10.3% in rivaroxaban compared with 11.4% in warfarin (HR = 0.90; 95%CI: 0.76-1.07).

Rivaroxaban in extended treatment was tested in EINSTEIN-extension[23] and included 1196 patient who had completed at least 6 mo of treatment in either the EINSTEIN-DVT or EINSTEIN PE study. It was a phase Ⅲ, randomized, double blind, placebo controlled study comparing an additional 6-12 mo of rivaroxaban 20 mg daily to placebo. Rivaroxaban significantly reduced the occurrence of recurrent VTE (1.3%) compared to placebo (7.1%) (HR = 0.18; 95%CI: 0.09-0.39), indicating superiority. This however, was at the expense of a small increase in bleeding with major bleeding occurring in 4 patients (0.7%) taking rivaroxaban compared with none in the placebo group. Clinically relevant non-major bleeding was also significantly greater with 5.4% in rivaroxaban and 1.2% in placebo.

These results highlight that rivaroxaban is non-inferior to warfarin with a potential reduction in major bleeding. As rivaroxaban does not require initial parenteral anticoagulation, it has the added benefit of simplicity and potential for early discharge and management in the community. In extended therapy, rivaroxaban was superior to placebo for the prevention of recurrent VTE, but also resulted in increased major and non-major bleeding.

Apixaban

Apixaban is small molecule inhibitor of factor Xa. It has an oral bioavailability of about 50%. Peak plasma levels are reached in about 3 h and like all other factor Xa inhibitors, the half life is short at about 12 h. Like rivaroxaban, apixaban is metabolized in the liver by CYP3A4 and CYP independent pathways. About 25% of apixaban is excreted renally with the rest excreted in faeces[22,25].

AMPLIFY[26] was the pivotal study that compared apixaban to standard therapy, with subcutaneous enoxaparin followed by warfarin, in the treatment of VTE. Apixiban was given at a higher dose of 10 mg twice daily for 7 d for initial treatment, followed by a lower dose of 5 mg twice daily for 6 mo. This was a phase Ⅲ, randomized, double blind study, on 5395 patients, most of whom had DVT (65.5%) with fewer having PE (25.2%) and PE with DVT (8.8%). Apixaban was non-inferior to standard therapy with recurrent VTE occurring in 2.3% in apixaban compared with 2.7% in the conventional group (HR = 0.84; 95%CI: 0.60-1.18). Major bleeding was reduced significantly in the apixaban group (0.6%) compared with standard treatment (1.8%) (HR = 0.31 95%CI: 0.17-0.55). There was also a significant reduction in major and clinically relevant non-major bleeding with a greater than 50% reduction in the apixaban group (4.3%) compared to standard treatment (9.7%) (HR = 0.44, 95%CI: 0.36-0.55).

AMPLIFY-EXT[27] was a randomized, double blind, placebo controlled study in 2486 patients who had completed at least 6 mo of anticoagulation as part of the AMPLIFY study. Two doses of apixaban (2.5 mg twice daily and 5 mg twice daily) were compared to placebo for 12 mo Recurrent VTE occurred in 8.8% of patients on placebo compared with 1.7% on apixaban 2.5 mg and 1.7% on apixaban 5 mg. The rates of major bleeding were low in all groups with placebo (0.5%), apixaban 2.5 mg (0.2%) and apixaban 5 mg (0.1%). Rates of clinically relevant non-major bleeding were also comparable in all groups with placebo (2.3%), apixaban 2.5 mg (3.0%) and apixaban 5 mg (4.2%).

These results highlight that, not only has apixaban been shown to be non-inferior compared with standard therapy with regards to efficacy, it is also associated with less major and non-major bleeding in the treatment of VTE. In fact, the risk of major and non-major bleeding appears to be comparable to placebo in extended therapy, making apixaban a unique and attractive option. Similar to rivaroxaban, there is no need for initial treatment with parental anticoagulation and thus may help facilitate early discharge and treatment in the community in low risk PEs.

Edoxaban

Edoxaban is an oral, direct, selective FXa inhibitor. It is rapidly absorbed with a peak concentration occurring within 1-3 h and is short acting with a half life of 9 to 11 h. It has linear pharmacokinetics within the therapeutic dose range and is not altered by food intake. It has a high oral bioavailability of about 50% which is comparable to the other FXa inhibitors. Edoxaban, like the other factor Xa inhibitors has a dual mechanism of excretion with 50% of edoxaban and its metabolites excreted renally; the remaining 50% is excreted in faeces[28].

Hokusai-VTE[29] was the pivotal study of edoxaban in VTE and was a phase Ⅲ randomized, double blind study of 8240 patients with DVT (60%) and PE (40%). All patients received a heparin bridge prior to commencing edoxaban 60 mg, edoxaban 30 mg (CrCl 30-50 mL or weight < 60 kg) or warfarin for 3 to 12 mo. Recurrent symptomatic VTE occurred in 3.2% in the edoxaban group compared with 3.5% in the warfarin group (HR = 0.89 95%CI: 0.70-1.13), proving edoxaban is non-inferior to warfarin. There was no significant difference in major bleeding, with 1.4% in edoxaban compared with 1.6% in warfarin (HR = 0.84, 95%CI: 0.59-1.21). There was however, statistically less non-major bleeding with 7.2% in edoxaban and 8.9% in warfarin (HR = 0.80 95%CI: 0.68-0.93).

This was the largest single phase Ⅲ study in novel oral anticoagulants in VTE and showed that edoxaban was non-inferior to warfarin in the prevention of recurrent symptomatic VTE. Edoxaban also caused

Table 1 Comparative pharmacology of novel oral anticoagulants

	Dabigatran	Rivaroxaban	Apixaban	Edoxaban
Target	IIa (thrombin)	Xa	Xa	Xa
Bioavailability	8%	66% without food 100% with food	50%	62%
Time to peak level (h)	1.25-3	2-4	1-4	1-2
Half life (h)	12-17	5-9 young 11-13 elderly	12	9-11
Trough	12-24	16-24	12-24	12-24
Drug interactions	Proton pump inhibitors	Potent CYP3A4 and P-glycoprotein inhibitors	Potent CYP3A4 and P-glycoprotein inhibitors	P- glycoprotein inhibitors
Renal excretion	80%	35%	25%	50%
Coagulation test effect	aPPT > 2 × ULN at trough → increased bleeding	Prolonged PT may indicate excess bleeding risk but local calibration required	PT and aPTT prolonged but no known relation to bleeding risk	PT and aPTT prolonged but no known relation to bleeding risk
	dTT at trough > 200 ng/mL or > 65 s → excess bleeding	Anti-FXa chromogenic assays: quantitative but no data on threshold values for bleeding or thrombosis	Anti-FXa chromogenic assays: quantitative but no data on threshold values for bleeding or thrombosis	Anti-FXa chromogenic assays: quantitative but no data on threshold values for bleeding or thrombosis

dTT: Diluted thrombin test.

Table 2 Phase 3 novel oral anticoagulant trials

	RECOVER and RECOVER II	EINSTEIN DVT	EINSTEIN PE	AMPLIFY	Hokusai VTE
Drug	Dabigatran	Rivaroxaban	Rivaroxaban	Apixaban	Edoxaban
N	5128	3449	4832	5395	8240
Indication	VTE	DVT	PE	VTE	VTE
Heparin bridge	Yes	No	No	No	Yes
Duration (mo)	6	3, 6, 12	3, 6, 12	6	3, 6, 12

DVT: Deep vein thrombosis; PE: Pulmonary embolism; VTE: Venous thromboembolism.

less bleeding overall however there was no difference in major bleeding compared with warfarin. Like dabigatran, this study used a heparin bridge as initial treatment, rendering treatment regimes more complex and less amenable for early discharge (Tables 1-3).

MONITORING OF NOACS

Routine monitoring is not required for the NOACs and dosing should not be changed in response to laboratory coagulation results. There may however, be special circumstances, such as urgent surgery or serious bleeding, whereby the quantitative assessment of anticoagulant effect may be required. Measures of drug exposure may also be useful if there is concern regarding poor compliance and recurrent thrombotic effects, as well as in the presence of renal or hepatic dysfunction.

Unlike VKA monitoring, it is critical that the timing of NOAC use relative to blood testing is known, as all the NOACs have a short duration of action with maximal effect occurring at the maximal plasma concentration. Traditional anticoagulation markers such as aPTT and PT may provide a qualitative assessment however, they cannot provide a quantitative assessment. For most clinical scenarios however, qualitative information may be sufficient as clinicians are mostly concerned about extremely high or low levels of anticoagulation.

Direct thrombin inhibitors

Dabigatran has almost no effect on PT and INR at clinically important concentrations and therefore these tests are not useful in determining the anticoagulant effect of direct thrombin inhibitors (DTIs). The aPTT may provide a qualitative assessment however the relationship between aPTT and dabigatran is curvilinear and results need to be interpreted with caution. In the presence of DTIs, there is a larger change in aPTT at lower concentrations but less significant change at higher concentrations. This is further complicated by the fact that the sensitivity of different aPTT reagents varies greatly. That said, a normal aPTT indicates no clinically relevant anticoagulation effect and an aPTT trough level that is above two times the upper limit of normal warrants caution, as it may be associated with a higher risk of bleeding[30].

Quantitative tests do exist but are not widely available in most hospitals as yet. Ecarin clotting time assay is a test that directly measures the activity of

Table 3 Efficacy and safety

	Incidence of recurrent VTE			Incidence of major bleeding		
	NOAC	Warfarin	HR (95%CI)	NOAC	Warfarin	HR (95%CI)
RE-COVER	2.4%	2.1%	1.10 (0.65-1.84)	1.6%	1.9%	0.82 (0.45-1.48)
RE-COVER II	2.3%	2.2%	1.08 (0.45-1.48)	1.2%	1.9%	0.69 (0.36-1.32)
RE-MEDY[1]	1.8%	1.3%	1.44 (0.78-2.64)	0.9%	1.8%	0.52 (0.27-1.02)
EINSTEIN DVT	2.1%	3.0%	0.68 (0.45-1.48)	0.8%	1.2%	0.65 (0.33-1.30)
EINSTEIN PE	2.1%	1.8%	1.12 (0.75-1.68)	1.1%	2.2%	0.49 (0.31-0.79)
AMPLIFY	2.3%	2.7%	0.84 (0.60-1.18)	0.6%	1.8%	0.31 (0.17-0.55)
HOKUSAI-VTE	3.2%	3.5%	0.89 (0.70-1.13)	1.4%	1.6%	0.84 (0.59-1.21)

[1]Extended treatment study. NOAC: Novel oral anticoagulant; DVT: Deep vein thrombosis; PE: Pulmonary embolism; VTE: Venous thromboembolism.

DTIs. When dabigatran is given twice daily, a trough level greater than 3 times normal is associated with a higher risk of bleeding[31]. A diluted thrombin test (dTT) is a test that requires calibration, but can predict the level of anticoagulation more accurately. Hemoclot is a dTT that, when used with the appropriate calibrators for dabigatran, provides a direct linear relationship between the anticoagulant effect and dabigatran concentration. A normal dTT implies no significant anticoagulant effect of dabigatran while a Hemoclot of > 200 ng/mL (equal to dTT > 65 s) at trough dabigatran plasma concentration, is associated with an increased risk of bleeding[31]. There is however no current data on a cut-off dTT below which surgery can be undertaken safely.

Factor Xa

The different FXa-inhibitors affect PT and aPTT to a varying extent, however neither provides quantitative results. There is a weak prolongation of aPTT with paradoxical response at low concentrations as well as significant variability with different assays, thus rendering aPTT an unacceptable measure of FXa inhibitory effect[32]. The effect on PT is concentration dependent and is more reflective of FXa inhibitory effect however this also depends on both the assay and the type of FXa inhibitor. Assay specific calibrators and calibration curves can be made for rivaroxaban, providing qualitative information on anticoagulant effect[33]. No such data is currently available for apixaban or edoxaban.

Anti-FXa "chromogenic assays" are new tests that have been developed to assess plasma concentrations of the FXa-inhibitors. Theses tests require validated calibrators and are becoming increasing available commercially. Biophen DiXal is an example of such an assay and gives in vitro quantitative measurements of direct FXa inhibitors on human citrated blood plasma. It is based on the inhibition of a constant amount of exogenous FXa and the hydrolysis of a FXa specific chromogenic substrate by the residual FXa. It is suitable to measure rivaroxaban plasma concentrations in a wide range and there is acceptable inter-laboratory precision[34]. Similar studies have also been performed with apixaban showing linear dose-response curves[35,36].

We can see that the thrombin times (aPTT and dTT) are affected by the DTIs whereas the Factor Xa inhibitors tend to prolong PT. These tests can provide qualitative assessment of anticoagulation but there are significant limitations in interpretation. While quantitative tests do exist, the major limitation, other than the availability of these tests, is that information regarding harmful and therapeutic ranges are currently lacking. The practical guide to use can see Table 1. Figure 1 shows regimes for anticoagulation in pulmonary embolism (Table 4).

CONCLUSION

Overall, 27048 patients have been involved in phase 3 trials into the efficacy and safety of NOACs in VTE. We can seen from the studies outlined above that all the NOACs are non inferior to warfarin with efficacy ranging between 1.8%-3.2% in the incidence of recurrent VTE in the NOAC arms and between 1.3%-3.5% in the warfarin arms. The studies have also shown either a trend or statistically significant reduction in bleeding compared with standard therapy with heparin and warfarin, with major bleeding ranging from 0.6%-1.4% in NOACs compared with 1.2%-2.2% with warfarin. Apixaban appeared to be associated with the lowest bleeding risk of all the NOACs and was comparable to placebo in extended treatment in terms of bleeding. Given that the length of anticoagulation beyond 3 mo should be determined by the risk of bleeding with anticoagulation, these results have the potential to significantly influence decisions regarding the length of treatment.

Another concept introduced by some of the NOACs is the option for no initial parenteral anticoagulation. NOACs have a fast onset of action so, unlike warfarin, parenteral anticoagulation is not a necessity. Rivaroxaban and apixaban were the only NOACs to provide an alternative regime, where instead a heparin, patients were given an increased dose of the same drug in the initial phase of treatment. This has the

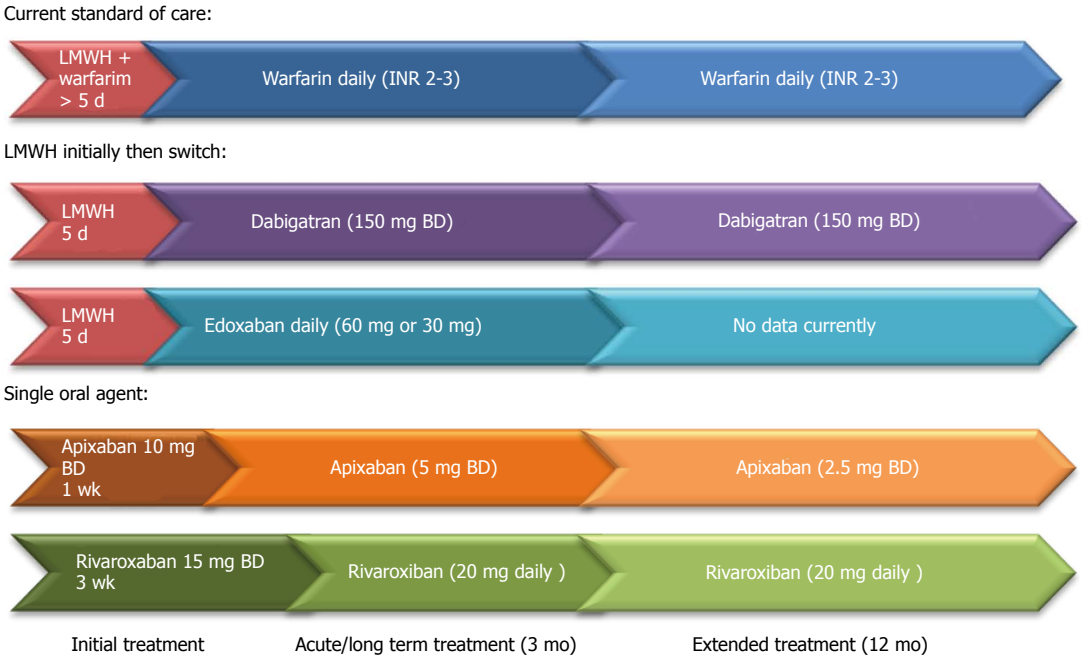

Figure 1 Regimes for anticoagulation in pulmonary embolism.

Table 4 Practical guide to use

When considering the use of a NOAC, there are important steps that should be considered:
(1) Consideration as to whether anticoagulation is necessary
Does the patient have a confirmed indication for anticoagulation?
Did the patient have a transient risk factor for VTE that has resolved or did they have an unprovoked VTE and should be considered for extended treatment?
(2) Consideration as to whether a NOAC is the most appropriate choice
Does the patient have normal renal and liver function?
Does the patient have an underlying malignancy for which LMWH may be a more appropriate alternative?
(3) Review of any other medications that may be contra-indicated or pose unfavourable drug-drug interactions
Potent inhibitors: ketoconazole, itraconazole, voriconazole, posaconazole
Potent inducers: rifampicin, carbamazepine, phenytoin, phenobarbital, HIV protease inhibitors
(4) Education regarding the importance of compliance and bleeding risk
Due to the short half life, there is a rapid decline in protective anticoagulation
(5) Regular follow-up to assess:
Therapy adherence
Potential thromboembolic event
Any adverse events
Bleeding events
Co-medications
Blood tests for haemoglobin, renal and hepatic function
(6) Assessment to determine whether ongoing anticoagulation is necessary and beneficial

NOAC: Novel oral anticoagulant; LMWH: Low molecular weight heparin; HIV: Human immunodeficiency virus; VTE: Venous thromboembolism.

potential to simplify treatment and facilitate early discharge and is reflected in the most recent ESC guideline, with early discharge and treatment at home recommended in patients with low risk PE.

While the results of these trials are very enticing, it important to remember that patient selection is critical when deciding to use these agents. Patients included in these trials were younger, with less co-morbidity and lower risks of bleeding than the patients usually seen in clinical practice. Patients with significant renal and hepatic dysfunction were excluded and there were few patients with strong indications for extended anticoagulation, namely cancer, antiphopholipid syndrome and recurrent VTE, included in these studies. Also, testing of anticoagulant effect is limited in its availability and correlation to risk. Careful consideration and more real world experience are therefore needed when using these new but promising treatments.

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CASE REPORT

Intravascular proliferating anaplastic lymphoma kinase-positive anaplastic large-cell lymphoma

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Author contributions: Shiroshita K and Imataki O contributed equally to this work; Shiroshita K and Imataki O wrote the manuscript; Kida J and Matsumoto K designed the research; Uemura M essentially contributed to the treatment of the case; Yamaoka G performed analysis; Miyai Y and Haba R contributed to the pathological analysis; and Imataki O organized the report.
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Conflict-of-interest: None.

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Abstract

An 82-year-old Japanese man visited our emergency unit complaining of dyspnea. Laboratory data showed 15% atypical lymphocytes in peripheral blood which expressed the T-cell phenotype. Chest/abdominal computed tomography depicted hepatosplenomegaly and swelling of systemic lymph nodes. The patient died of advanced respiratory failure 5 d after the first occurrence of his dyspnea. At autopsy, the pathological features revealed a diffuse infiltration of large atypical lymphocytes to systemic organs including the spleen and lung. In immunohistochemical staining, these cells expressed CD30, TIA-1, anaplastic lymphoma kinase (ALK), CD5 and CD3. An advanced surface molecule analysis revealed a lack of CD54 (intercellular cell adhesion molecule-1) and CD56 (neural cell adhesion molecule). We observed the proliferation and infiltration of these lymphoma cells specifically at the intravascular lesions similar to intravascular lymphoma (IVL). T-cell IVL is not established as an independent clinical entity in the World Health Organization classification, and our patient's ALK-positive T-IVL in lung appears to be the first reported case.

Key words: Malignant lymphoma; Cytotoxic molecule; Intravascular lymphoma; Anaplastic lymphoma kinase; Anaplastic large-cell lymphoma

Core tip: Intravascular lymphoma (IVL), known as a mature B-cell neoplasm that proliferates intravascularly, is established as an independent clinical entity in the World Health Organization classification. Our patient's case suggests that IVL is one of the characteristic proliferation patterns commonly seen in lymphomas including B- and T-cell neoplasms, anaplastic large-cell lymphoma, and more. We speculate that the function of the original lymphocytes (T or B cells) designates the tumor biology

of IVL. Intravascular proliferation is determined by a lack of adhesion molecules. We suspect that the biological aggressiveness would be modified by other characteristic phenotypes such as anaplastic lymphoma kinase, CD30, and CD56.

Shiroshita K, Kida J, Matsumoto K, Uemura M, Yamaoka G, Miyai Y, Haba R, Imataki O. Intravascular proliferating anaplastic lymphoma kinase-positive anaplastic large-cell lymphoma. *World J Hematol* 2015; 4(2): 10-15 Available from: URL: http://www.wjgnet.com/2218-6204/full/v4/i2/10.htm DOI: http://dx.doi.org/10.5315/wjh.v4.i2.10

INTRODUCTION

B-cell intravascular lymphoma (B-IVL) is a rare and special subtype of diffuse large B-cell lymphoma (DLBCL). B-IVL is characterized by the selective growth of lymphoma cells within the lumina of vessels. Intravascular large B-cell lymphoma is categorized as a distinct clinical entity in the World Health Organization (WHO) classification[1,2]. The lymphoma cells are widely disseminated in and involved with extranodal sites, but lymph nodes are usually exclusively spared. The sites of involvement vary and may include organs such as bone marrow, liver, spleen, lung, brain and skin. IVL tumor cells are immunophenotypically positive for mature B-cell markers such as CD19, CD20, CD21 and CD79a, but negative for the homing receptors CD29 (β1 integrin) and CD54 [intercellular adhesion molecule 1 (ICAM-1)]. These molecular features of the cell surface are thought to hinder the extravasated infiltration of tumor cells.

A few anecdotal cases of intravascular T-cell lymphoma (T-IVL) have been reported[3,4]. In these cases the tumor cells expressed T-cell markers, but not the differentiated markers CD4 and CD8. CD30 was also positive. Pleomorphic morphology resulted in the diagnosis of anaplastic large-cell lymphoma. Among these T-IVL cases, none harbored the gene of anaplastic lymphoma kinase (ALK). Here we report the first case of a patient with ALK-positive T-IVL, and we suggest a distinct clinical entity for T-IVL and propose the elucidation of its clinicopathological features.

CASE REPORT

An 82-year-old Japanese man with no notable medical history came to the ambulatory emergency unit of our hospital because of difficulty in breathing that had persisted for the prior 4 d. In addition to his chief complaint of dyspnea, he had been suffering from anorexia due to nausea, and diarrhea. The dyspnea was progressive and became urgent just 2 h prior to his admission to our emergency unit. A physical examination showed that he was febrile (38.1 ℃). His blood pressure was within the normal range at 132/85 mmHg, but tachycardia (136/min) was observed. The respiration rate was 31 breaths/min and irregular. The oxygen saturation was 90% while he was breathing ambient air.

An electrocardiogram revealed arrhythmia at a rate of 130-140 beats/min, with premature atrial contraction and nonsustained ventricular tachycardia, and there was no evidence of ST-segment or T-wave abnormalities. An echocardiogram revealed MR (grade Ⅱ) and TR (grade Ⅰ) and hypokinesia in the septum, the posterior wall, and the base of the heart; the ejection fraction was 60% and there was no evidence of systolic function disorder.

A laboratory examination revealed an elevated white blood cell (WBC) count of 24260/μL and an increase of 13% atypical lymphocytes in the WBC differential count. In a blood smear, the atypical lymphocytes were large round cells and their cytosol was basophilic; they also had some vesicles. Anemia with a hemoglobin level of 12.5 g/dL and thrombocytopenia with a platelet count of 10.8/μL were observed. The C-reactive protein level was 32.0 mg/dL, *i.e.*, extremely elevated, and there were slight impairments of liver and renal functions. The prothrombin time international normalized ratio was long, and the d-dimer (7.4 μg/dL) and fibrinogen degradation product (26.8 μg/dL) levels were both elevated. The arterial blood gas test revealed pH 7.46 and metabolic acidosis, accompanied by an elevated level of lactic acid (86 mg/dL), which was compensated for by respiratory alkalosis.

Soluble interleukin-2 receptor was extremely over-secreted (up to 231900 U/mL). The antigens of *Streptococcus pneumonia* and *Legionnaire* in the urine and the cultures of blood, sputum, and urine were all negative, as were hepatitis B virus surface antigen and hepatitis C virus antibody. The residual examination findings were intact. X-ray photography elucidated bilateral pleural fluid, an enlarged mediastinum, and swelled bilateral hilar lymph nodes. Chest and abdominal computed tomography confirmed the findings suggested on chest X-ray and depicted hepatosplenomegaly and swelling of the hilar, mediastinum, supraclavicular, and left axial lymph nodes. For the management of the patient's respiratory and circulatory failure, we admitted him to our intensive care unit.

Unfortunately, the patient died of respiratory failure 5 d after the initiation of dyspnea[5]. At autopsy, the pathological features revealed a diffuse infiltration of large atypical lymphocytes to systemic organs including lymph nodes (Figure 1), lung and heart. In the lung and heart, tumor cells selectively proliferated in the intra lumina of vessels (Figure 2). Immunohistochemical staining revealed that the tumor cells expressed CD30, TIA-1, ALK (Figure 1), CD5 and CD3. Surface markers for cell adhesion, *i.e.*, CD54 (ICAM-1) and CD56 (neural cell adhesion molecule), were negative. Both epithelial membrane antigen and Epstein-Barr virus (EBV)-encoded small RNA were also negative. Interestingly, granzyme B and perforin were positive. Ki-67 was

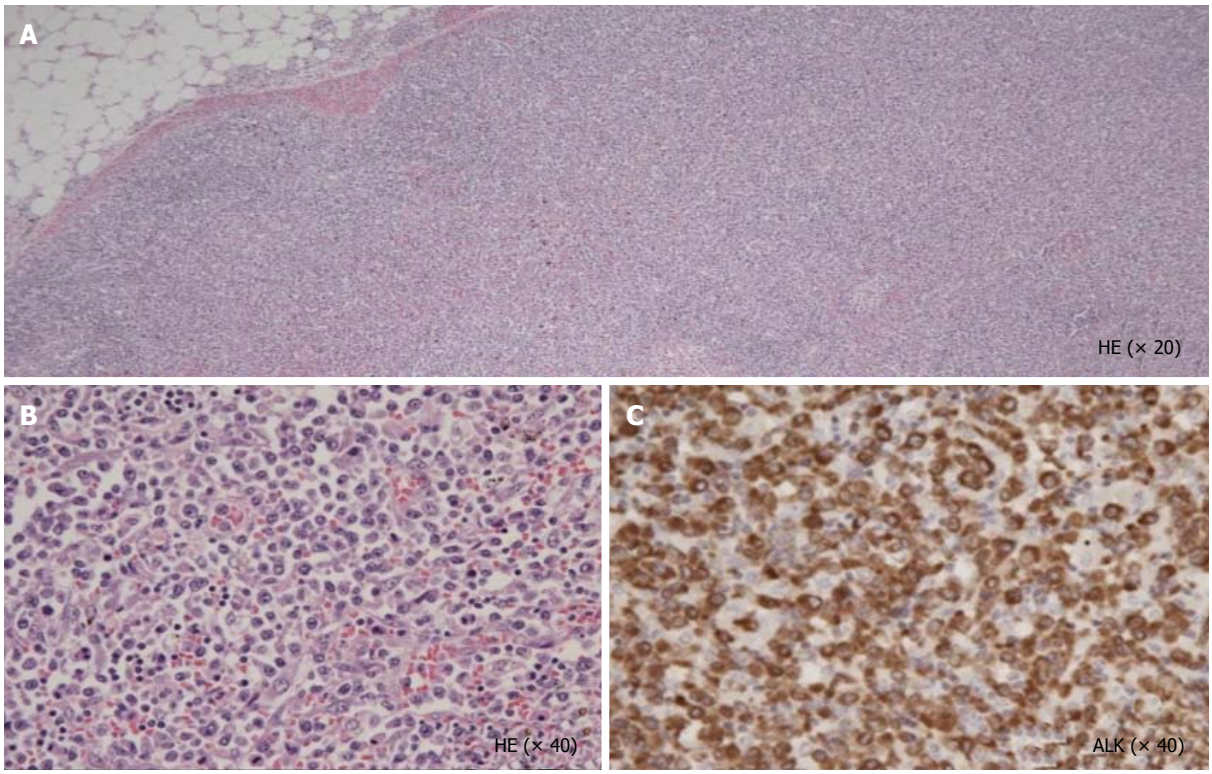

Figure 1 Autopsy was performed. Hematoxylin-eosin staining in lymph node revealed diffuse proliferation of tumor cells in low power field (A); High power field imaging showed infiltrated atypical small lymphocytes (B); Immunohistochemical staining revealed that the tumor cells expressed anaplastic lymphoma kinase (C). ALK: Anaplastic lymphoma kinase.

strongly positive.

DISCUSSION

IVL was first described as an angiotropic large-cell lymphoma in which neoplastic lymphoma cells proliferate exclusively within the lumen of small blood vessels[6]. The majority of IVL cases have a B-cell phenotype; other lymphoma phenotypes including T cells[3,7], and NK cells[8] are rare. T-cell lymphoma with IVL proliferation is nevertheless the second most common phenotype of IVL, followed by the NK-cell subtype. IVL has been reported to be linked to cases of plasmablastic lymphoma[9] and even histiocytic[10] and Hodgkin lymphoma[11]. The aggregate of these cases indicates that IVL can compromise all types of lymphoid malignancies (lymphoid neoplasms) or hematological malignancies, and indeed a genetic analysis of IVL revealed the neoplastic cells to be of lymphoid origin[6,12].

IVL defined by the WHO criteria includes "the presence of neoplastic lymphocytes only in the lumina of small vessels, particularly capillary"[1,2]. Lymphocyte migration from a blood vessel to tissues is remarkably important for the immune response in the inflammatory area. Therefore, based on the immunobiological behaviors of the lymphocytes, it has been assumed that IVL cells can proliferate and grow mainly in small vessels. However, the mechanism underlying the endotheliotropic invasion of IVL neoplastic cells is not fully elucidated.

One of the possible explanations for the underlying mechanism was suggested by the results of some B-IVL studies that analyzed the lack of homing receptors (CD44), LFA-1 (CD11a/18), and ICAM-1 (CD54)[2,13] and CD 29 (β1 integrin) and CD54 (ICAM-1)[14,15] on neoplastic cells. In our patient's case, ICAM-1 was not expressed. This is consistent with the past reports of B-IVL. Akamatsu *et al*[16] reported adhesion molecules in CD30-positive anaplastic large-cell lymphoma (ALCL), and they revealed that homing receptors were constantly expressed and ICAM-1 was frequently expressed. These findings suggest that the tumor cells of B-IVL and T-IVL may have a common mechanism of selective proliferation in blood vessels. However, the lack of cell adhesion molecules is only part of a hypothesis regarding the tumorigenesis of IVL, and it is not clear whether the lack of adhesion molecules is important for IVL.

There is the possibility that tumor cells actively proliferate in the vessels; for example, the overexpression of other adhesion molecules or surface proteins may make the tumor cells stay in the vessels. To elucidate the etiology of IVL, a further accumulation of cases in which the adhesion molecules are examined is necessary. If its etiology is revealed, IVL may be classified as a new etiology in the WHO classification, such as cell adhesion molecules lacking lymphoma, or CD54-negative lymphoma.

As a cause of neoplastic transformation, an association

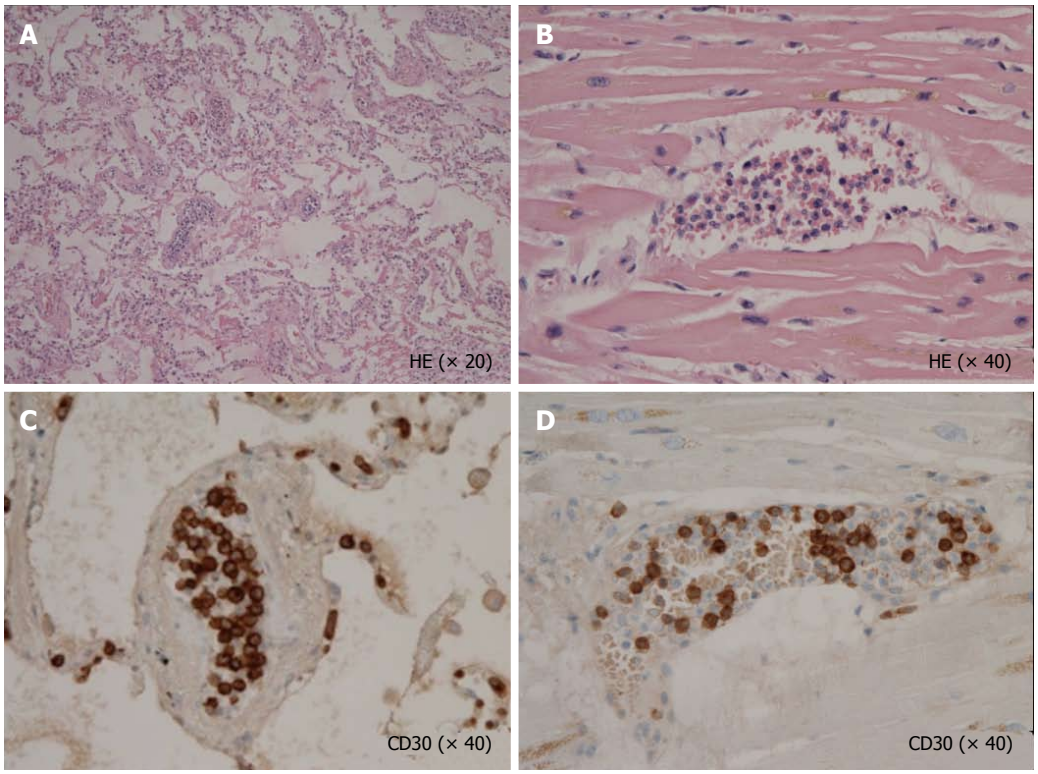

Figure 2 Anaplastic lymphoma kinase positive tumor cells were selectively proliferating in intra lumina of vessels in lung (A, C) and heart (B, D), respectively.

of EBV has been pointed out in the past IVL cases[8]. Among lymphoid neoplasms, an EBV association has been observed in DLBCL, peripheral T-cell lymphoma-unspecified (PTCL-U), and extra-nodal NK/T cell lymphoma-nasal type[8]. Our patient's case revealed no association with EBV. According to past T-IVL case reports, the EBV association is observed only in some cases[8]. More T-IVL cases should be accumulated to determine the significance of EBV in terms of prognosis and response to chemotherapy, and for a comparison of the nature of the original lymphoma and its intravascular type. Interestingly, NK-IVL showed EBV in most cases[17,18], which suggests the possibility that the mechanism of malignant transformation in IVL may be the same as that of the original lymphoma.

Ko *et al*[19] reported a first case of intravascular ALCL in lung in 1997. However, they did not report the case's ALK expression. Several subsequent reports of intravascular ALCL showed that ALK was negative in almost all cases[7,20,21]. Thus, ALK expression is not essential for the etiology of ALCL IVL; however, the ALK phenotype might modify the clinical grade of tumor growth[4,19]. The question of whether cases of ALK-positive ALCL IVL have poorer clinical outcomes compared to ALK-negative ALCL IVL is a topic for further research. Likewise, the biological role of cytotoxic molecules might be also associated with the aggressive behavior of ALCL IVL.

Takahashi *et al*[3] reported a CD30-positive and ALK-negative intravascular ALCL case, which was the first report of intravascular ALCL with cytotoxic molecules. The present case strongly expressed TIA-1, granzyme B and perforin, all of which are cytotoxic molecules, and thus our patient's case is the second with cytotoxic molecules. Krishnan *et al*[4] reported the first case of ALK-positive intravascular ALCL in 2009 in breast skin; however, the case did not show an intravascular proliferation pattern in any visceral organs. To our knowledge, therefore, the present case is the first report of ALK-positive ALCL in lung presenting as IVL. Our patient's case is the first of intravascular ALCL which simultaneously expressed ALK-positive and cytotoxic molecules.

The significance of cytotoxic molecule expression is controversial. In a PTCL-U study, 20% of the lymphomas expressed cytotoxic molecules such as TIA-1 and granzyme B in lymphoma-associated hemophagocytic syndrome (HPS)[22]. In our patient's case, we incidentally detected HPS in the bone marrow, although the criteria of Asian-variant IVL (which was originally established for B-IVL) were not met. These findings suggest that intravascular ALCL that expresses cytotoxic molecules has an aggressive clinical course or is frequently concurrent with lymphoma-associated HPS. In contrast, HPS is frequently observed in B-IVL, especially among the reported Asian-variant cases, which manifested hepatosplenomegaly, pancytopenia and pathological HPS[23]. It would be interesting to conduct a hematopathology study of the cytotoxic molecules of the Asian variant and compare them with those seen in T-IVL.

In summary, we treated a patient with intravascular

proliferating ALK-positive ALCL with cytotoxic molecules. T-IVL is substantially different from B-IVL in clinical aspects, but not immunophenotypically[24]. We speculate that the original T-cell function and migration reflect the tumor biology of T-IVL[25]. Indeed, whichever malignant lymphoma is harboring the intravascular component, it still retains the clinical and biological features of each original subtype of malignant lymphoma[8]. To elucidate the differences of clinical features between B-IVL and T-IVL, further investigation is mandatory. Many cases of IVL may be identified and reported in the future; not only B-IVL (a subtype of DLBCL) but also T-IVL, NK-IVL, and other IVL-like lymphomas. The further accumulation of cases and studies will contribute to the establishment of the etiology of IVL.

COMMENTS

Case characteristics

An 82-year-old Japanese man with no remarkable medical history visited the emergency unit complaining of severe dyspnea.

Clinical diagnosis

The patient was febrile and presented with shortness of breath.

Differential diagnosis

Malignant lymphoma, lymphoblastic leukemia and other mature lymphoid neoplasms.

Laboratory diagnosis

Laboratory data showed 15% atypical lymphocytes in peripheral blood, and the phenotypical analysis of the atypical lymphocytes, which expressed CD2, CD4, CD30, and intracellular CD3 antigen.

Imaging diagnosis

Chest and abdominal computed tomography depicted hepatosplenomegaly and swelling of hilar, mediastinal, supraclavicular, and left axial lymph nodes.

Pathological diagnosis

A diffuse infiltration of large atypical lymphocytes to systemic organs including the spleen and lung and immunohistochemical staining revealed that these cells expressed CD30, TIA-1, anaplastic lymphoma kinase (ALK), CD5, CD3 and a lack of CD54 [intercellular adhesion molecule 1 (ICAM-1)] and CD56 (neural cell adhesion molecule).

Treatment

The patient did not receive any treatment for lymphoma and he died of advanced respiratory failure.

Related reports

The etiology of intravascular lymphoma has been understood as that of a mature B-cell neoplasm which is immunophenotypically negative for homing receptors such as CD29 (β1 integrin) and CD54 (ICAM-1). The majority of intravascular lymphoma (IVL) cases have a B-cell phenotype. Some anecdotal cases of intravascular T-cell lymphoma (T-IVL) have been reported, but few cases of intravascular ALK-positive anaplastic large-cell lymphoma have been reported.

Term explanation

Anaplastic large-cell lymphoma (ALCL) is a common subtype of T-cell lymphoma. The homing receptors are cell adhesion molecules targeting endothelial cells and constantly expressed in lymphocytes, even in malignant lymphoma subtypes such as ALCL.

Experiences and lessons

T-IVL is substantially different from B-cell IVL (B-IVL) in clinical aspects, but not immunophenotypically. The tumor cells of B-IVL and T-IVL may have a common mechanism of selective proliferation in blood vessels due to a lack of homing receptors, and we speculate that the original lymphocyte function reflects the tumor biology of IVL.

Peer-review

The authors presented a case of an 82-year-old man with IVL from ALK-positive anaplastic large cell lymphoma. It caused respiratory failure in his lungs and he also had splenomegaly.

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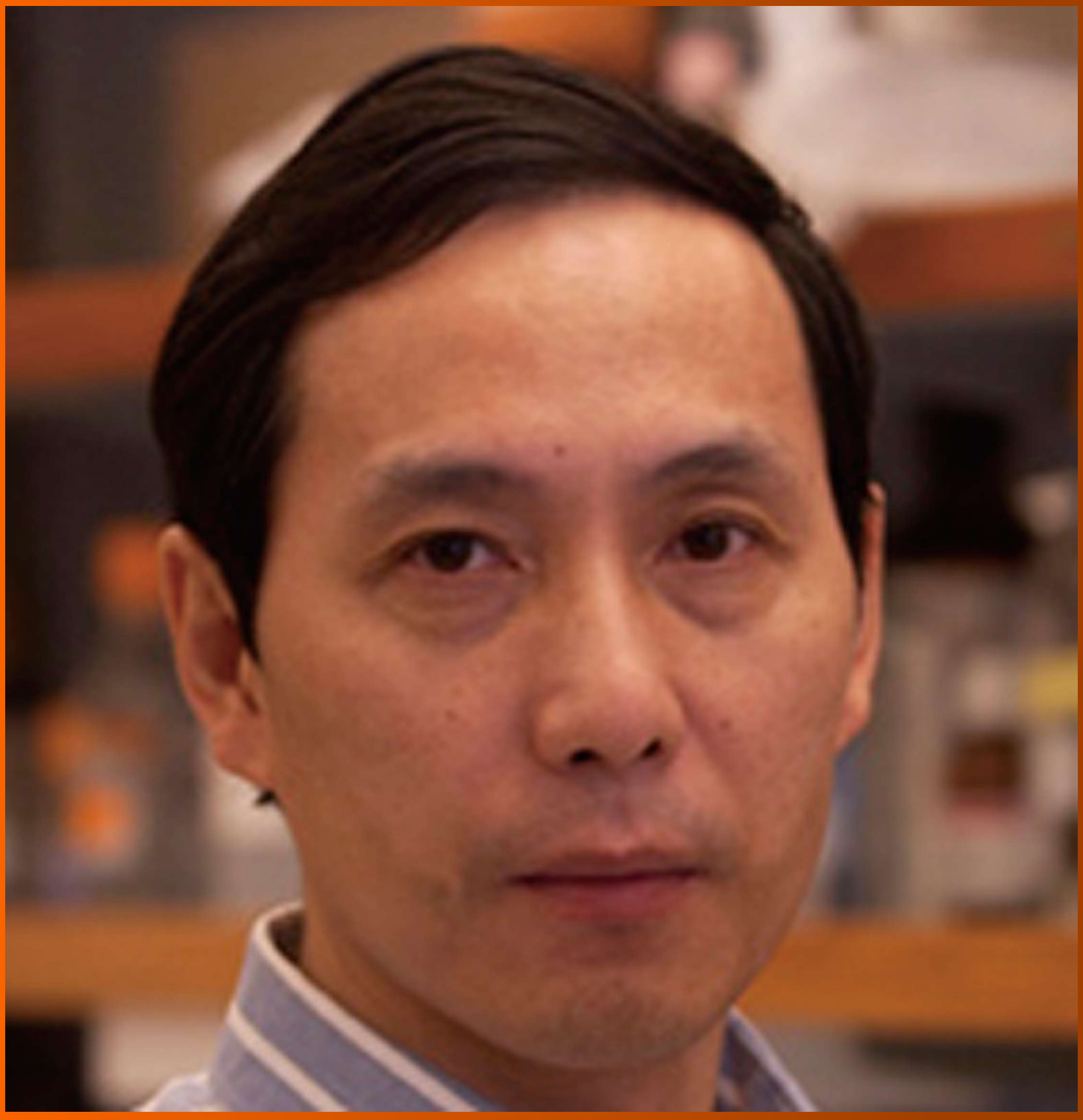

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REVIEW

European *vs* 2015-World Health Organization clinical molecular and pathological classification of myeloproliferative neoplasms

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Abstract

The *BCR/ABL* fusion gene or the Ph[1]-chromosome in the t(9;22)(q34;q11) exerts a high tyrokinase acticity, which is the cause of chronic myeloid leukemia (CML). The 1990 Hannover Bone Marrow Classification separated CML from the myeloproliferative disorders essential thrombocythemia (ET), polycythemia vera (PV) and chronic megakaryocytic granulocytic myeloproliferation (CMGM). The 2006-2008 European Clinical Molecular and Pathological (ECMP) criteria discovered 3 variants of thrombocythemia: ET with features of PV (prodromal PV), "true" ET and ET associated with CMGM. The 2008 World Health Organization (WHO)-ECMP and 2014 WHO-CMP classifications defined three phenotypes of $JAK2^{V617F}$ mutated ET: normocellular ET (WHO-ET), hypercelluar ET due to increased erythropoiesis (prodromal PV) and ET with hypercellular megakaryocytic-granulocytic myeloproliferation. The $JAK2^{V617F}$ mutation load in heterozygous WHO-ET is low and associated with normal life expectance. The hetero/homozygous $JAK2^{V617F}$ mutation load in PV and myelofibrosis is related to myeloproliferative neoplasm (MPN) disease burden in terms of symptomatic

splenomegaly, constitutional symptoms, bone marrow hypercellularity and myelofibrosis. JAK2 exon 12 mutated MPN presents as idiopathic eryhrocythemia and early stage PV. According to 2014 WHO-CMP criteria JAK2 wild type MPL^{515} mutated ET is the second distinct thrombocythemia featured by clustered giant megakaryocytes with hyperlobulated stag-horn-like nuclei, in a normocellular bone marrow consistent with the diagnosis of "true" ET. JAK2/MPL wild type, calreticulin mutated hypercellular ET appears to be the third distinct thrombocythemia characterized by clustered larged immature dysmorphic megakaryocytes and bulky (bulbous) hyperchromatic nuclei consistent with CMGM or primary megakaryocytic granulocytic myeloproliferation.

Key words: Myeloproliferative disorders; Essential thrombocythemia; Primary megakaryocytic granulocytic myeloproliferation; Myelofibrosis; JAK2^{V617F} mutation; MPL^{515} mutation; Calreticulin mutation; JAK2 wild type; Myeloproliferative neoplasm; Bone marrow pathology; Polycythemia vera

Core tip: The 2015 World Health Organization-Clinical Molecular and Pathological criteria define three phenotypes of JAK2^{V617F} mutated myeloproliferative neoplasms (MPNs) essential thrombocythemia (ET), prodromal polycythemia vera (PV), prodromal PV, hypercellular megakaryocytic-granulocytic myeloproliferation and classical PV *vs* the JAK2 exon 12 mutated idiopathic eryhrocythemia and PV. MPL^{515} mutated JAK2 wild type ET and myelofibrosis is a distinct thrombocythemia without features of PV in blood and bone marrow. Calreticulin mutated JAK2/MPL wild type ET and myelofibrosis is the third thrombocythemia entity with characteristic features of primary megakaryocytic granulocytic myeloproliferation in the bone marrow, which are not seen in JAK2 and MPL mutated MPNs. MPN disease burden is best reflected by the degree of anemia and splenomegaly on top of mutation allele burden, bone marrow cellularity and increase of reticulin fibrosis.

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INTRODUCTION

Dameshek *et al*[1] proposed in 1940 a set of symptoms, signs and laboratory tests for the diagnosis of polycythemia vera (PV) based on the description of 20 cases with PV seen between 1928 and 1937. Dameshek believed that the following minimal data should be present before a definite diagnosis of PV can be made: plethoric appearance, splenomegaly, definitely elevated erythrocyte count above 6 × 10^{12}/L, elevated platelet count, and elevated hematocrit. The bone marrow is pathognomonic diagnostic showing a panmyelosis (increased trilinear hematopoiesis) and large megakaryocytes[1,2]. In a doubtful case, the procedure of blood volume estimation may be helpful[1-3]. Between 1940 and 1950, Dameshek[2] considered the majority of PV patients as fundamentally normal[3]. The PV patient frequently has a long life span and every attempt should be made to keep the treatment of PV as physiologic as possible by venesection aiming at hematocrit of 0.40 as a satisfactory method resulting in a state of iron deficiency[1-3]. Red cell count remains elevated above 6 × 10^{12}/L due to microcytosis of red cells, but hematocrit and hematocrit levels remain low for periods of months to years. During of complete remission of PV by phlebotomy alone the patient is reasonable asymptomatic indicating that the best index of phlebotomy therapy is the hematocrit value and hematocrit concentration[2,3]. During the state of chronic iron deficiency and normal values of haematocrit (0.40), the patient himself frequently becomes completely asymptomatic. On this program it is possible to control PV patients for several up to ten to fifteen years and such PV are in as good health as comparable persons of the same age group. Dameshek hesitated to use a potentially dangerous radioactive material in an individual with a relatively long life span and questioned whether the acute leukemic states in some cases are due to the potentially leukemogenic drug P^{32} or may be part of the natural history of PV. In the experience of Dameshek in about 50 reasonably well followed cases of polycythemia, acute leukemia developed in only 1 (2%) instance without previous roentgen ray or radioactive phosphor therapy[3-5].

Dameshek[2] (1900-1969, Figure 1) defined in 1950 PV as a total marrow disorder in which peripheral blood erythrocytosis, leukocytosis and thrombocytosis are all simultaneously present and the bone marrow is featured by a trilinear myeloproliferative disease (MPD) of erythrocythemia, thrombocythemia and granulocythemia[2,3]. PV is complicated by primary myeloid metaplasia of the spleen with increasing degree of splenomegaly, myelofibrosis and the development of anemia in about one third of the cases after long-term follow-up of about 15 to 30 years. Dameshek[2] proposed in 1950 the one cause hypothesis cause for PV as a trilinear MPD due to the presence of excessive bone marrow stimulation by an unknown factor or the lack or diminution of an inhibitory factor[2,3]. The one cause hypothesis of Dameshek for trilinear PV has been confirmed by Vainchenker in France by the discovery in 2005 of the acquired somatic JAK2^{V617F} mutation as the cause of three phenotypes of MPD essential thrombocythemia (ET), PV and myelofibrosis (MF)[2,3].

In this historical appraisal of the MPDs and myelo-

Table 1 The 1980 Rotterdam clinical and pathological criteria for essential thrombocythemia and polycythemia vera

1A The 1980 RCP major (A) and confirmative (B) criteria for prefibrotic ET		
A1 Persistent platelet count in excess of 400 × 10^9/L		
A2 Increase and clustering of enlarged megakaryocytes in bone marrow biopsy		
A3 No or slight increase of reticulin fibers (RF 0 or RF 1)		
B1 Presence of large platelets in a peripheral blood smear		
B2 Absence of any underlying disease for reactive thrombocytosis and normal ESR		
B3 No splenomegaly (< 12 cm) or slight splenomegaly on palpation or scan (< 15 cm)		
B4 Increase of LAP-score and no signs of fever or inflammation		
Exclusion criterion		
Ph^+ chromosome and any other cytogenetic abnormality in blood or bone marrow cells		
1B The 1980 RCP major (A) and minor (B) criteria for prefibrotic PV		
A1 Raised red cell mass. Male > 36 mL/kg, female > 32 mL/kg consistent with erythrocyte count of > 6 × 10^{12}/L (Dameshek[1,2])		
A2 Absence of primary or secondary erythrocytosis by clinical and laboratory tests		
A3 Slight, moderate or marked increase in bone marrow biopsy of clustered, enlarged pleomorphic megakaryocytes with hyperlobulated nuclei and moderate to marked increase cellularity of megakaryopoiesis/erythropoiesis or typically trilinear mega-erythro-granulopoiesis. A typical PV bone marrow excludes erythrocytosis. No or presence of reticuline fibers and no collagen fibers (no dry tap)		
B1 Thrombocythemia, persistant increase of platelet > 400 × 10^9/L		
B2 Leukocytosis, leucocyte count > 10^9/L and low erythrocyte sedimentation rate		
B3 Raised leukocyte alkaline phosphatase score > 100, absence of fever or infection		
B4 Splenomegaly on palpation or on isotope/ultrasound scanning		
A1 + A3 plus one of B establishes PV and excludes any variant of erythrocytosis		
1C Grading of bone marrow biopsy content of RF according to Ellis *et al*[41], Georgii *et al*[35,36] and Wilkins *et al*[171] and WHO grading of MF[98-101]		
Grading RF[41]	Grading MF 2008 WHO[91]	Description of RF and reticulin/collagen fibers in MF as a secondary event in MPN
Normal RF 0	N MF 0	No reticulin fibers, occasional individual fibers or focal areas with tiny amount of reticulin fiber network
Slight increase RF 1	+ MF 0	Fine reticulin fiber network throughout much of section and no course reticulin fibers
Moderate increase RF 2	++ MF 1	Diffuse fine reticuline network with focal collections of thick course reticulin fibers and no collagenisation
Marked increase RF 3	+++ RCF = MF 2	Diffuse and dense increase in reticulin with extensive intersections, and presence of collagen fibers and no or minor O
OS Dry tap RF 4	Sclerotic RCF and O = MF 3	Diffuse and dense reticulin with with coarse bundles of collagen associated with significant O

MF: Myelofibrosis; MPN: Myeloproliferative neoplasms; O: Osteosclerosis; RCP: Rotterdam clinical and pathological; ET: Essential thrombocythemia; RF: Reticulin fibrosis; PV: Polycythemia vera.

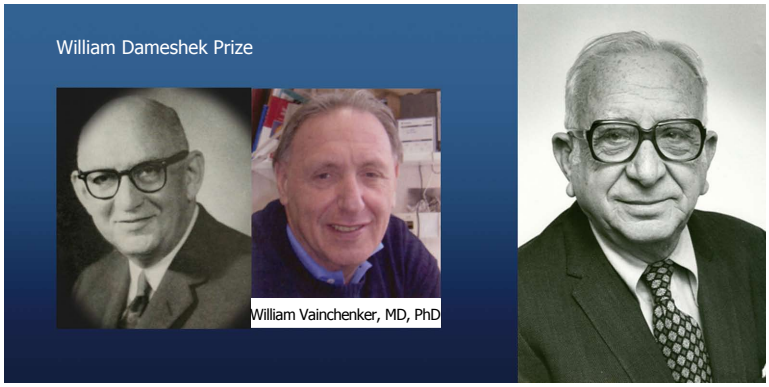


Figure 1 Dameshek 1900-1969. Description of trilinear PV in 1950. "Photo courtesy of the American Society of Hematology (ASH) 2012." Louis Wasserman 1912-1999. Founder of the polycythemia vera study group 1967. Photo by Jerry Soalt. "Courtesy of the ASH" 2012. William Vainchenker discoverer of the JAK2V617F mutation in 2005 as the cause of the trilinear MPNs ET, PV and MF. MF: Myelofibrosis; PV: Polycythemia vera; ET: Essential thrombocythemia; MPN: Myeloproliferative neoplasms.

proliferative neoplasm (MPN) the Rotterdam, Hannover, Cologne and European clinical and bone marrow criteria *vs* the polycythemia vera study group (PVSG) and World Health Organization (WHO) classifications (Tables 1-7) are compared against the 2015 WHO-Clinical Molecular and Pathological (CMP) criteria for the diagnosis and staging of the masked and manifest MPNs ET, PV and MF (Tables 8-12).

MYELOPROLIFERATIVE DISORDERS *VS* CHRONIC MYELOID LEUKEMIA

In 1951 Dameshek[6] illogically proposed an unifying theory that erythroleukemia, chronic myeloid leukemia (CML), PV, idiopathic or agnogenic myeloid metaplasia (AMM) of the spleen, megakaryocytic leukemia or primary represent one myeloproliferative activity of bone

Table 2 Polycythemia vera study group criteria for polycythemia vera[10] and diagnostic differentiation of polycythemia vera from all variants of primary and secondary erythrocytoses by bone marrow histology[17]

Major criteria PV	Minor criteria PV
A1 RCM, males > 36 mL/kg females > 32 mL/kg. Hemoglobin 18.5 g/dL male and > 16.5 g/dL females (PVSG, WHO)	B1 Thrombocytosis Platelet count > 400 × 10^9/L
A2 Normal arterial oxygen saturation > 92%	B2 Leukocytosis > 12 × 10^9/L
A3 Splenomegaly on palpation	B3 Raised neutrophil alkaline phosphatase score > 100 or raised B12 (> 900 ng/L) or raised unsaturated B12 binding capacity (> 2200 ng/L)
	B3 is replaced by spontaneous EEC as a specific clue to PV
Benign erythrocytosis: 1980 RCP criteria	Myeloproliferative PV: 1980 RCP criteria
RCM, males > 36 mL/kg females > 32 mL/kg or increased erythrocytes above 6 × 10^{12}/L	Increased red cell counts > 6 × 10^{12}/L or increased RCM and increase of clustered large megakaryocytes with hyperlobulated nuclei is a pathognomonic diagnostic clue to PV
Normal platelet and leukocyte counts	Normal RCM = inapparent erythrocytosis is not associated with splenomegaly and shows normal bone histology, whereas IPV is associated with splenomegaly and show typical features of PV bone marrow histology
Normal bone marrow histopathology: normal cellularity and erythropoiesis, and normal size, morphology and distribution of megakaryocytes	
Classification of erythrocytoses[74]	Notes anno 1980-1999[74]
Congenital or primary erythrocytosis including mutation truncated EPO recepotor, disrupted oxygen homeostasis in Chuvash erythrocytosis, high oxygen affinity hemoglobinopathy, and congenital autonomous EPO production	Increased RCM does not distinguish between PV and primary erythrocytosis
	Increased RCM does not distinguish between PV and IPV. IPV is featured by advanced PV with normal hb, Ht and erythrocyte count due to splenomegaly and hypersplenism and with increase of reticulin fibrosis with typical PV bone marrow features
Secondary erythrocytosis due to autonomous EPO production in renal diseases or by tumour cels or due to hypoxia	In IPV the values of hemoglobin hematocrit and erythrocytes are normal but RCM is increased due to splenomegaly with absence of hypervolemic symptoms
Idiopathic erythrocytoses	

Diagnostic criteria for polycythemia vera (PV) proposed in 1975 by the polycythemia vera study group (PVSG)[10] and used in the 2001 and 2008 World Health Organization Classifications of MPD and MPN[75,76]. Diagnosis of PV is acceptable if the following combinations are present: A1 + A2 + A3 or A1 + A2 + any two from category B. Diagnostic differentiation of benign erthrocytosis from myeloproliferative polycythemia vera by histopathology from bone marrow sections according to the Rotterdam clinical and pathological (1980 RCP criteria for PV) (Table 1)[73,74]. RCM does not distinguish between PV and primary or secondary erythrocytosis. RCM does not distinguish between PV and inapparent PV with splenomegaly in splanchnic vein thrombosis (IPV, Table 3). In IPV RCM is increased due to splenomegaly with absence of hypervolumemic symptoms. Bone marrow histology distinguishes PV from all variants of erythrocytosis with a sensitivity and specificity of 100%. RCM: Raised red cell mass; EEC: Erythroid colony formation; IPV: Inapparent PV.

Table 3 Comparion of clinical and laboratory features between polycythemia vera study group defined polycythemia vera (group A) and inapparent polyctemia vara (group B)

Clinical feature	Group A PV	Group B IPV	*P*-value
No. of cases	85	18	
Age (range)	61 (27-83)	52 (28-82)	NS
Sex male/female	56/42%	39/61%	NS
Splenomegaly	44 (52%)	15 (83%)	< 0.005
Leukocytes > 12 × 10/L	31 (36%)	5 (28%)	NS
Platelets > 500 × 10/L	40 (47%)	10 (56%)	NS
Red cell counts × 10/L: males	6.2 (4.9-7.4)	5.2 (4.7-5.9)	< 0.0002
Red cell mass males	48.2 (36-60)	43.3 (41-61)	NS
Red cell counts females	6 (4.2-7.3)	4.7 (3.7-5.5)	< 0.003
Red cell mass females	40.1 (32-59)	37.3 (34-46)	NS
Plasma volume PV *vs* IPV			
Increase/theoretical norm (%)	10 (-11, 61)	36 (20, 98)	< 0.00001

Lamy *et al*[44]. Conclusion: inapparent polycythemia vara (IPV) is featured by normal values of haemoglobin, haematocrit and erythrocyte counts, in the absence of hypervolumic symptoms and in IPV red cell mass is increased related to the degree of increased spleen size. NS: Not significant.

marrow cells due to one hypothetical stimulus[6,7] on the basis of which the PVSG defined in 1975 the authorative criteria for the disgnosis of PV, AMM and primary hemorrhagic thrombocythemia (PHT)[8-10]. PMF or AMM is a clinicopathological entity not preceded by any other PVSG defined MPD PHT or ET, PV, CML or preleukemia with myelodysplastic features. CML is leukemia or neoplasia that destroy normal hematopoiesis whereas ET, PV and AMM form a benign proliferation of trilinear hematopoietic proliferation in the bone marrow (myeloproliferation) and extramedullary hematopoiesis in the spleen[8].

Lumping of erythroleukemia with PV, and putting together chronic granulocytic or myeloid leukemia with PV was without scientific foundation[6,7,11]. Dameshek *et al*[11] and Dameshek[12] (1990-1969) separated in 1969 erythroleukemia from CML and PV by describing that all variations of the chronic and acute erythroleukemias form a distinct entity, the Di Guglielmo syndrome[11,12]. When running its full course, the Di Guglielmo syndrome appeared to pass through three stages of refractory anemia with predominant erythroid hyperplasia and maturation arrest with development of dysplastic features and gradual transition into a mixed erythroblastic myeloblastic leukemia. According to a prospective clinical basic research study by Michiels[13] and Michiels *et al*[13,14], the sequential preleukemic stages of the Di Gugliemo syndrome appeared to be a continuum of trilinear myelodysplatic syndrome (MDS), refractory anemia with excess of blasts (RAEB) and acute myeloid leukemia (AML)[13,14].

DISCOVERY OF BCR/ABL IN PHILADELPHIA-POSITIVE CHRONIC MYELOID LEUKEMIA

Nowell *et al*[15] discovered a disease specific minute cytogenetic marker in patients with CML, labelled after

Table 4 Clinical and hematological findings in thrombocythemia of various myeloproliferative diseases polycythemia vera, primary myelofibrosis and essential thrombocythemia in 395 myeloproliferaive disease patients from the Cologne Institute of Pathology 1980-1989

Diagnosis cologne criteria	PV	PMF	True ET	NV
No. of patients	55	250	40	
Thrombocythemia > 500 × 10^9/L (%)	48	48	100	< 350
Thrombocythemia > 1000 × 10^9/L (%)	6	17	65	
Age (median years)	63	66	58	
Male/female	20/35	58/62	14/26	
Platelets, × 10^9/L mean ± SD	808 ± 288	960 ± 361	1386 ± 541	< 350
Erythrocytes, × 10/L mean ± SD	6.7 ± 0.2	4.5 ± 0.1	4.6 ± 0.7	< 6.0
Hemoglobin, g/dL	17.7 ± 0.4	12.8 ± 0.2	13.7 ± 2	
Leukocytes	17 ± 1	15 ± 9	13 ± 5	
Leukocyte alkaline phosphatase score	164 ± 91	98 ± 83	57 ± 43	< 10
Spleen size increase on palpation (cm)	2.0 ± 3.3	2.6 ± 3.1	0.4 ± 0.8	NP
Observed 10 yr survival (mo)	106	85	170	
Specific loss of life expectancy (%)	19	22	3	
Bone marrow histopathology	PV	PMF	True ET	RT
Megakaryocytes	Pleomorph	Immature giant	Staghorn	N
Frequency/mm hematopoietic area	123/27	112/37	157/45	98/39
Size (μm^2)	385 ± 102	386 ± 197	425 ± 117	328 ± 84
Erythropoiesis × 10	44 ± 8	9 ± 4	22 ± 5	27 ± 4
Granulopoiesis × 10	65 ± 12	58 ± 27	47 ± 15	57 ± 18
Reticulin fibers × 10 (mm)	21 ± 11	97 ± 41	15 ± 7	15 ± 6
Early-prefibrotic pCIMF	Early prefibrotic	ET	NV	
No. of patients	120	40		
LAP score	110 ± 60	57 ± 43	< 100	
Spleen size	1.7 ± 1.4	0.4 ± 0.8	NP	
Observed survival (mo)	77	170	-	
Specific loss in life expectancy (%)	53	3	0	

Data from Thiele *et al*[90]. MPD: Myeloproliferaive disease; PV: Polycythemia vera; PMF: Primary myelofibrosis; ET: Essential thrombocythemia; LAP: Leukocyte alkaline phosphatase; RT: Reactive thrombocytosis; NV: Normal value; NP: Not palpable; N: Normal.

the city of discovery the philadelphia (Ph^1). With the advent of the Philadelphia chromosome (Ph^1) as a disease specific marker for CML patients, Gilbert[16] of the PVSG separated in 1973 Ph^1-positive CML from the Ph^1-negative MPDs PV, ET and AMM[16,17]. Using improved banding techniques, Janet Rowley (1973, Figure 2) showed that the Ph^{1+} chromosome in CML represents a deletion of the long arm of chromosome 22 (22q-) resulting in the minute Ph^{1+} chromosome (Figure 3)[18]. Additional studies showed that a large part of 22q was translocated to 9q, and that a small part of 9q was translocated to 22q resulting in the translocation (t) t(9;22)(q34;q11)[19,20].

The discovery that the translocation t(9;22) in the Ph^{1+} chromosome resulted in the *BCR/ABL* translocation in the early 1980s originates from the search by three Dutch investigators Nora Heisterkamp, John Groffen and Gerard Grosveld (Figure 2)[21-24]. John Groffen and Nora Heisterkamp obtained their Drs degree in Groningen and moved to the United States in 1981 and worked in John Stephenson's lab in Frederick to study viral oncogenes. Gerard Grosveld was working at the Erasmus University in Rotterdam on a project to identify the Ph^{1+}-chromosome breakpoint. They worked together at the Erasmus University Rotterdam (EUR) and Erasmus Medical Center (EMC), Rotterdam, and at the National Health Institute in Frederick, MD United States (personal communications Gerard and Frank Grosveld 2008-2012). The *BCR/ABL* discovery runned through a three step scientific process: (1) John Groffen learned to make cosmid libraries in Dick Flavell's lab in the MRC in London and took the technique along to the United States. There John Groffen and Nora Heisterkamp cloned parts of the human *ABL* gene and in collaboration with Walter Bodmer's group in the United Kingdom localized *ABL* to chromosome 9. Using a v-abl probe Heisterkamp and Groffen had localized *ABL* on human chromosome 9[21]; (2) Groffen and Heisterkamp contacted Gerard Grosveld mediated by Frank Grosveld and collaborated. Using somatic cell hybrids made by Anne Hagemeijer, (chief of Medical Cytogentics EMC), they found *c-ABL* moved to the Ph^1 -chromosome. Using hybrid cell lines containing the segregated Philadelphia translocation products (generated by Dr. Ad Geurts van Kessel, EUR), Groffen, Heisterkamp and Gerard Grosveld investigated whether *cABL* moved from the long arm of chromosome 9 to the long arm of the Ph^1 chromosome by Southern blot analysis[22]. Indeed c-*ABL* was found to translocate to the Ph^1-chromosome even in patients with complex chromosomal translocations but not in Ph^1-negative CML patients with apparently normal karyotypes[23]; and (3) John Groffen and Nora Heisterkamp cloned more to the 5' of *ABL* and discovered and cloned a breakpoint fragment

Table 5 The 2002 European Clinical and Pathological criteria for the diagnosis of "true" essential thrombocythemia and chronic idiopathic myelofibrosis or primary megakaryocytic granulocytic myeloproliferation according to Michiels *et al*[91]

Clinical ECP criteria of "true" ET	Pathological ECP criteria of "true" ET
A1 Persistent increase of platelet count grade 1 400-1500 × 10^9/L, grade 2 > 1500 × 10^9/L	B1 Predominant proliferation of enlarged to giant megakaryocytes wit hyperlobulated staghorn-like nuclei and mature cytoplasm, lacking conspicious cytological abnormalities
A2 Normal spleen or only minor splenomegaly on echogram	B2 No proliferation or immaturity of granulopoisis or erythropoiesis
A3 Normal LAP score, normal ESR and increased MPV	B3 No or only borderline increase in reticulin fibers
A4 Spontaneous megakaryocyte colony formation (CFU-Meg)	The combination of A1 and B1 + B2 establish "true" ET. Any other criterion confirms ET
A5 No signs or cause of reactive thrombocytosis	
A6 No preceding or allied other subtype of MPN, PV, MDS or CML	
A7 Absence of Philadelphia chromosome	
Clinical ECP criteria of CIMF or PMGM	Pathological ECP criteria of CIMF or PMGM
A1 No preceding or allied other subtype of MPN, PV, CML or MDS	B1 PMGM and relative or absolute reduction of erythropoiesis (erythroid precursors). Abnormal clustering and increase of atypical immature medium-sized large to giant megakaryocyte containing (Cloud-like) hypolobulated nucle and definitive maturation defects
Early clinical stage	Staging of myelofibrosis: MF in ET, PV and PMGM
Normal hemoglobin, or anemia grade 1: Hemoglobin > 12 g/dL, slight or moderate splenomegaly on palpation or > 11 cm on ultrasound or CT. Thrombocythemia around 1000 × 10^9/L	MF 0 No reticulin fibrosis RF 0/1
	MF 1 Slight reticulin fibrosis RF 2
	MF 2 Marked increase RF grade 3 and slight to moderate collagen fibrosis
	MF 3 Advanced collagen fibrosis-osteosclerosis (endophytic bone formation)
Intermediate clinical stage	
Anemia grade 2, hemoglobin > 10 g/dL, definitive leuko-erythroblastic blood picture and/or tear-drop erythrocytes. Splenomegaly on palpation, no adverse signs	
Advance clinical stage	
Anemia grade 3, hemoglobin < 10 g/dL, significant splenomegaly and one or more adverse signs	

ECP: European Clinical and Pathological; CIMF: Chronic idiopathic myelofibrosis; PMGM: Primary megakaryocytic granulocytic myeloproliferation; ET: Essential thrombocythemia; LAP: Leukocyte alkaline phosphatase; ESR: Erythrocyte sedimentation rate; MPV: Mean platelet volume; MPN: Myeloproliferative neoplasm; PV: Polycythemia vera; MDS: Myelodysplastic syndrome; RF: Reticulin fibrosis; CML: Chronic myeloid leukemia.

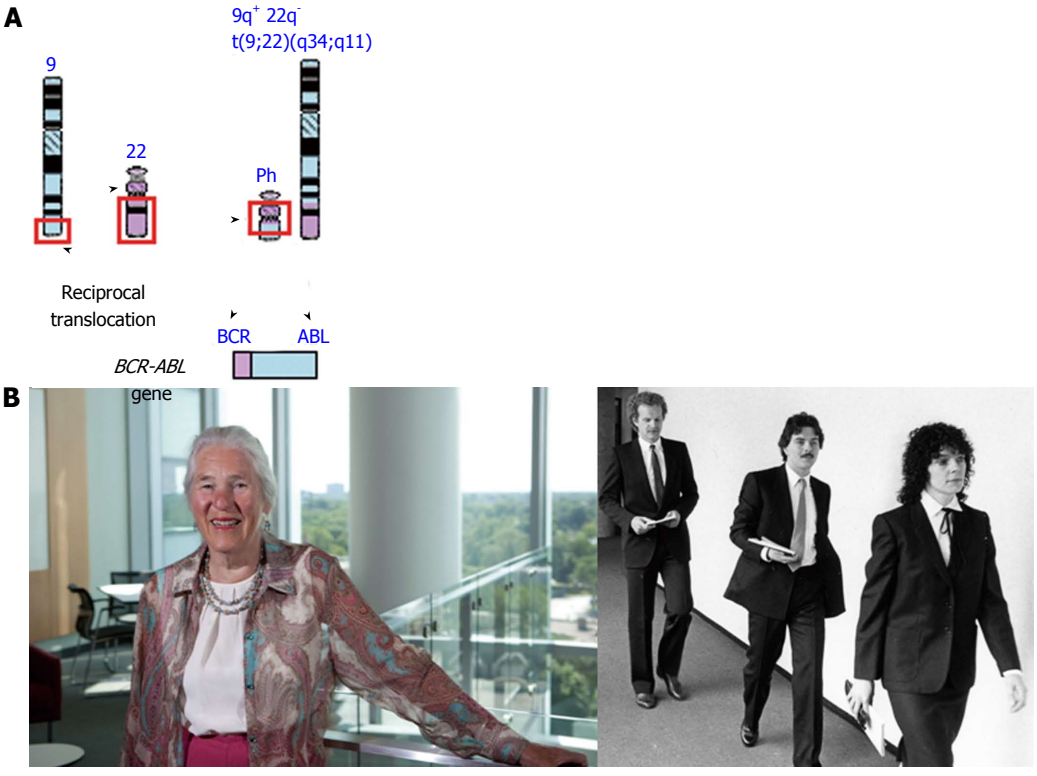


Figure 2 Reciprocal translocation t(9;22)(q34;q11) creates a novel breakpoint cluster region/ABL fusion gene with high tyrosinase activity as the cause of chronic myeloid leukemia. A: Dr. Janet Rowley, discoverer of t(9;22)(q34;q11) in Ph1chromosome positive chronic myeloid leukemia (CML)[19,20], and winner the Beutler Prize 2011, American Society of Hematology (ASH). Photo courtesy of ASH 2012; B: The discoverers of *BCR/ABL* fusion gene as the cause of Ph+ CML[21-24]. From left behind to right Gerard Grosveld, John Groffen and Nora Heisterkamp.

Table 6 The 2000 European Clinical and Pathological criteria for the diagnosis of polycythemia vera defined by Michiels[73] in 1997[9,74], and in 2000[93]

Clinical ECP criteria of PV	Pathological ECP criteria of PV
A1 Increased erythrocytes > 6 × 10^{12}/L. Raised RCM: RCM (optional) male > 36 mL/kg, female > 3.2 mL/kg or increased red cell counts above 6 × 10^{12}/L	B1 Thrombocytheia: platelet count > 400 × 10^{9}/L
A2 Absence of any cause of primary or secondary erythrocytosis by clinical and laboratory investigations	B2 Granulocytes > 10 × 10^{9}/L and raised LAP score in the absence of fever or infection
A3 Histopathology of bone marrow biopsy	B3 Splenomgaly on palpation or on echogram > 11 cm
(1) Increase and clusters of pleomorph large megakaryocytes with hyperploid nucei	B4 Spontaneous erythroid colony formation in the absence of EPO and low plasma or serum EPO level
(2) Increased cellulartity due to increased erythropoiesis or erythropoiesis and granulopoiesis (panmyelosis)	Staging of MF related to reticulin fibrosis
(3) No or slight increase of reticulin fibers	MF 0 No reticulin fibrosis RF 0/1
	MF 1 Slight reticulin fibrosis RF 2
	MF 2 Marked increase RF grade 3 and slight to moderate collagen fibrosis
	MF 3 Advanced collagen fibrosis-osteosclerosis

Diagnosis of idiopathic erythrocythemia (IE), early prodromal polycythemia vera (PV), classical PV or latent primary myeloproliferative disease (subclinical PV) are acceptable when[73,74,93]: A1 + A2 + A3 and none of B (except B4) is consistent with early erythrocythemic PV, labelled as idiopathic erythrocythemia: IE; A3 + B1 + B4 is consistent with prodromal PV with PV features in the bone marrow; A1 + A2 + A3 + plus one of B1 to 3 is consistent with classical PV; B4 confirms all variants of IE, prodromal PV and classical PV; A3 + B3 and none of the others is consistent with latent subclinical primary myeloproliferative diease (PMD) usually preceding masked cases of PV or MF during long term follow-up. ECP: European Clinical and Pathological; MF: Myelofibrosis; RF: Reticulin fibrosis; EPO: Erythropoetin; RCM: Red cell mass; LAP: Leukocyte alkaline phosphatase.

Table 7 2015 World Health Organization Clinical Molecular and Pathological criteria for the diagnosis of prodromal, masked and classical JAK2 mutated polycythemia vera *vs* primary or secondary erythrocytoses[77,78]

CM criteria	Bone marrow pathology (P) criteria (WHO)
Major criteria for PV	P1 Bone marrow pathology: increased cellularity (60%-100%) due to trilinear increase of erythropoiesis, megakaryopoiesis and granulopoiesis and clustering of small to giant (pleomorph) megakaryocytes with hyperlobulated nuclei
A1 Hematocrit > 0.51/> 0.48 in male/female Erythrocytes > 5.8 × 10^{12}/L males > 5.6 × 10^{12}/L females	Absence of stainable iron. No pronounced inflammatory reaction
A2 Presence of heterozygous and/or homozygous JAK2V617F or JAK2 exon 12 mutation	P2 Erythrocytosis. Normal erythropoiesis, normal granulopoiesis and megakaryocytes of normal size, morphology and no clustering
A3 Low serum Epo level	Grading of RF and MF
Minor	Prefibrotic: RF-0/1 = MF-0
B1 Persistent increase of platelet count × 10^{9}/L: grade 1: 400-1500, grade 2: > 1500	Early fibrotic: RF-2 = MF-1
B2 Granulocytes > 10 × 10^{9}/L or Leukocytes > 12 × 10^{9}/L and raised LAP-score or increased CD11b expression in the absence of fever or infection	Fibrotic: RCF 3 = MF-2
	Post-PV MF: RF 4 = MF-3
B3 Splenomegaly on ultrasound echogram (> 12 cm length in diameter) or on palpation	
B4 Spontaneous EEC formation (optional)	

2015 WHO-CMP criteria for staging of prodromal, erythrocythemic, and advanced polycythemia vera (PV). A2 + B1 + P1 establish early PV (mimicking ET) prodromal PV CMP stage 0; A1 + A2 + A3 + P1 and none of B establish idiopathic erythrocythemia (IE) or stage 1 PV; A1 + A2 + A3 + P1 and one or more of B establish classic stages of PV stage 2 and 3; A2 + B3 + P1 detect masked cases of PV with splenomegaly and hypersplenism to be labelled as inapparent PV (IPV) frequently seen Budd-Chiari syndrome or splanchnic vein thrombosis. CM: Clinical and molecular; RF: Reticulin fibrosis; MF: Myelofibrosis; ET: Essential thrombocythemia; WHO-CMP: World Health Organization clinical molecular and pathological; EEC: Endogenous erythroid colony.

from a CML patient DNA. Subsequent chromosome walking upstream from *ABL* identified a probe that recognized the chromosome 9 breakpoint in the DNA of a CML patient. Cloning of this fusion fragment provided probes of the breakpoint cluster region on chromosome 22, which detected the Philadelphia breakpoints in almost all CML patient samples including those with complex cytogenetic translocations. In CML patients, the chromosomal breakpoints were clustered within a limited region on chromosome 22, the "breakpoint cluster region": *BCR*[24]. The specific molecular *BCR/ABL* translocation on chromosome 22 in the t(9;22) of Ph[1]-positive CML patients was predicted to have functional significance for the disease[24]. There was no serendipity in the discovery of the *BRC/ABL* translocation t(9;22) and "they all were very lucky to have had their unique collaboration" (personal communication Nora Heisterkamp 2012).

The sequential discoveries of the Ph[1]-chromosome in the t(9;22)(q34;q11), and the *BCR/ABL* fusion gene on chromosome 22 became the cause of a clearly defined human neoplasia, *BCR/ABL* positive CML (Figure 3).

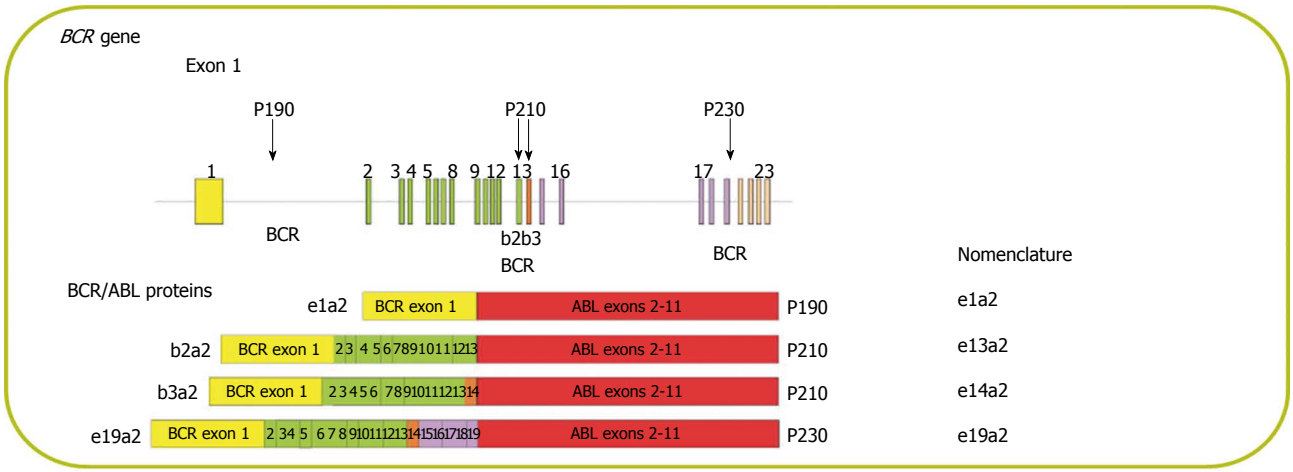


Figure 3 Locations of the breakpoint regions in the *BCR* gene and nomenclature of breakpoint cluster region/ABL proteins. BCR: Breakpoint cluster region.

Table 8 2015 World Health Organization clinical molecular and pathobiological criteria for diagnosis of JAK2^{V617F} mutated essential thrombocythemia[77,78]

CM criteria	Bone marrow pathology (P) criteria (WHO)
ET	Normocellular ET
(1) Platelet count of > 350 × 10^9/L and the presence of large platelets in a blood smear	Proliferation and clustering of enlarged mature pleomorphic megakaryocytes with hyperlobulated nuclei and mature cytoplasm, lacking conspicuous morphological abnormalities
(2) Heterozygous JAK2^{V617F} mutation, and low JAK2 allele mutation load	Normocellular bone marrow (< 60%) and no proliferation or immaturity of granulopoiesis or erythropoiesis
(3) Normal erythrocytes < 5.8 × 10^{12}/L males, < 5.6 × 10^{12}/L females	RF 0 or 1
(4) Hb and ht normal or in the upper range of normal	
Prodromal PV	ET with bone marrow features of PV
(1) Platelet count of > 350 × 10^9/L Hb and Ht normak or in the upper range of normal, normal erythrocyte < 5.8 × 10^{12}/L males, < 5.6 × 10^{12}/L females	Increased cellularity (60%-80%) due to increased erytropoiesis or trilineage myeloproliferation (*i.e.*, panmyelosis). Proliferation and clustering of medium sized to large (pleomorphic) mature megakaryocytes
(2) Presence of JAK2^{V617F} mutation and variable JAK mutation load	Absence bone marrow features consistent with congenital polycythemia and secondary erythrocytosis
(3) Low serum EPO level and increased LAP score	RF 0 or 1
(4) Spontaneous EEC	
Prefibrotic hypercellular ET	EMGM
(1) Platelet count of > 350 × 10^9/L	Hypercellular ET due to chronic megakaryocytic and EMGM and normal or reduced erythroid precursors
(2) Presence of JAK2^{V617F} mutation and high JAK2 mutation load	Loose to dense clustering of more pleiomorphic megakaryocytes with hyperploid or clumpsy nuclei (not or some cloud-like)
(3) Slight or moderate splenomegaly on ultrasound or on palpation	
(4) No preceding or allied CML, PV, PMGM, RARS-T or MDS	
Clinical stage 1: No anemia with Hb and Ht in the normal or low normal range: hb > 12 g/dL, normal LDH and CD34$^+$	Grading of reticulin fibrosis and MF in EMGM
Clinical stage 2: Slight anemia Hb < 12 to > 10 g/dL, LDH↑, and splenomegaly	Prefibrotic: RF 0/1 = MF 0, no/minor splenomegaly
Clinical stage 3: Anemia, Hb < 10 g/dL, LDH↑↑, CD34$^+$, leukoerythroblastose and, tear drop	Early fibrotic EMGM: RF 2 = MF 1 and minor or moderate splenomegaly
	Fibrotic EMGM: RF 3, RCF = MF 2 and overt splenomegaly
	Post-ET MF: RF 3/4 = MF 2/3 (WHO criteria)

ET: Essential thrombocythemia; MF: Myelofibrosis; EPO: Erythropoetin; CM: Clinical and molecular; Hb: Hemoglobin; ht: Hematocrit; WHO: World Health Organization; PV: Polycythemia vera; EPO: Erythropoetin; EEC: Endogenous erythroid colony; CML: Chronic myeloid leukemia; PMGM: Primary megakaryocytic granulocytic myeloproliferation; MDS: Myelodysplastic syndrome; EMGM: ET with hypercellular megakaryocytic-granulocytic myeloproliferation; RF: Reticuline fibrosis; LDH: Lactodehydrogenase; RCF: Reticulin collagen fibrosis.

The *BCR/ABL* fusion gene is detectable in hematopoietic bone marrow cells but not in fibroblasts of CML patients indicating that reticulin fibrosis in Ph-positive CML and ET is a reactive and secondary process. The *BCR/ABL* fusion gene produces a *BCR/ABL* protein, which has a high tyrosine kinase activity and CML-transformation capacity

Table 9 2015 WHO Clinical Molecular and Pathological criteria for the diagnosis of normocelular essential thrombocythemia carrying one of the MPL^{515} mutations[78]

CM JAK2 wild type ET	Bone marrow pathology (P) criteria (WHO)
(1) Platelet count > 350 × 10^9/L and presence of large platelets in blood smear	P1 Proliferation of large to giant mature megakaryocyte with hyperlobulated, staghorn-like nuclei in a normocellular bone marrow (< 65%)
(2) Hemoglobin, haematocrit and erythrocyte count in the normal range	No increase of erythropoiesis, and no increase or immaturity of granulopoiesis or erythropoiesis, no or slight increase in reticulin RF 0/1
(3) Presence of MPL^{515} mutation and JAK2 wild type	ET → MF
(4) Normal serum EPO	Increased reticulin fibrosis around dense clustered megakaryocytes in a normocellular bone marrow and reduced erythropoiesis. Follow-up data of RF and MF related to splenomegaly in MPL^{515} ET transltional states to MF are lacking. Grading of RF and MF similar as described for PV
(5) Normal LAP score and CD11b expression	
(6) No or slight splenomegaly	
(7) No leukoerythroblastosis	
(8) No preceding or allied CML, PV, RAS-T or MDS	

This entity is identical to "true" ET as defined in 2002 by Michiels and Thiele in Table 5[91]. ET: Essential thrombocythemia; CM: Clinical and molecular; RF: Reticulin fibrosis; MF: Myelofibrosis; EPO: Erythropoetin; LAP: Leukocyte alkaline phosphatase; CML: Chronic myeloid leukemia; PV: Polycythemia vera; MDS: Myelodysplatic syndrome; WHO: World Health Organization.

Table 10 World Health Organization-clinical molecular and pathological criteria for hypercellular essential thrombocythemia associated with primary megakaryocytic, granulocytic myeloproliferation caused by calreticulin mutations[78]

Clinical CM criteria JAK2 wild type PMGM	Pathological ECP criteria of CALR MGM
A1 No preceding or allied other subtype of myeloproliferative neoplasm PV, CML, MDS. The main presenting features is pronounced isolated thrombocythemia with platelet count around or above 1000 × 10^9/L	P1 PMGM and relative or absolute reduction of erythropoiesis and erythroid precursors. Abnormal dense clustering and increase in atypical medium sized, large to giant immature megakaryocytes containing bulbous (cloud-like) hypolobulated nuclei and definitive maturation defects
A2 Presence of CALR mutation and JAK2 wild type	
C Clinical stages of CALR MGM	MF Grading RF, MF
C1 Early clinical stage: Hb > 12 g/dL, slight to moderate splenomegaly, thrombocytosis around or above 1000 × 10^9/L, normal LAP score	MF 0 Prefibrotic CALR MGM, no reticulin fibrosis RF 0/1
C2 Intermediate clinical stage: slight anemia Hb < 12 to > 10 g/dL, decreasing platelet count, splenomegaly, increased LDH and definitive tear drop erythrocytes	MF 1 Early fibrotic CALR MGM slight reticulin fibrosis RF 2
C3 Advanced stage: anemia Hb < 10 g/dL, tear drop erythrocytes, increased LDH, increased CD34$^+$ cells, pronounced splenomegaly, normal or decreased platelet counts, leucocytosis or leukopenia	MF 2 Fibrotic CALR MGM increase RF grade 3 and slight to moderate collagen fibrosis
	MF 3 Advanced fibrotic CALR MGM with collagen fibrosis-osteosclerosis

The combination of A1 + A2 and P1 establishes calreticulin (CALR) ET and various clinical stages(C1, C2, C3) related to the degree of myelofibosis (MF). This entity has been defined as CMGM in the 1990 Bone Marrow Classification by Georgii *et al*[35] as the third MPD entity and as chronic idiopathic myelofibrosis false ET or prefibrotic or primary megakaryocytic granulocytic myeloproliferation in 2002 by Michiels and Thiele (Table 5)[91]. PMGM: Primary megakaryocytic, granulocytic myeloproliferation; RF: Reticulin fibrosis; CM: Clinical and molecular; PV: Polycythemia vera; MDS: Myelodysplatic syndrome; LAP: Leukocyte alkaline phosphatase; CML: Chronic myeloid leukemia; Hb: Hemoglobin; LDH: Lactodehydrogenase; MGM: Megakaryocytic granulocytic myeloproliferation.

in animal models[25-27]. Ninety percent of all CML patients are Ph^{1+}/*BCR/ABL*$^+$, 5% are Ph^{1-}/*BCR/ABL*$^+$, and 5% are Ph^{1-}/*BCR/ABL*$^-$, the latter group usually diagnosed as atypical CML, juvenile CML, chronic neutrophilic leukemia or chronic myelomonocytic leukemia[28]. The current molecular diagnosis of CML is made by peripheral blood reversed transscript PCR for the B*CR/ABL* fusion gene. Possible BCR fusion transscripts include P190, two variants of P210, and P230 and to be labeled as e1a2, e13a2, e14a2 and e19a2 (Figure 3). More than 98% of CML patients have P210 BCR transscript, of which one third e13a2 en two third 3 e14a2, and only 1% to 2% P190 BCR transscript having a poorer prognosis. The European LeukemiaNet recommendations for the diagnosis staging and management of CML patients are recently reviewed in great detail[29].

According to strict morphological, biochemical, cytogenetic and molecular criteria, Ph-positive CML is a malignant disease with an obligate transition into acute myeloid, lymphoblastic or megakaryoblastic leukemia, whereas ET, PV and AMM or chronic primary myelofibrosis (PMF) form the *BRC/ABL* negative MPDs featured by a benign proliferation of the three hematopoietic cell lines with a low incidence of leukemic transformation in PV and AMM[30-32]. In the Rotterdam cohort of 50 MPD patients seen between 1975 and 1985, Michiels and Hagemeijer found that all MPD patients diagnosed as ET, PV and MF were negative for the Ph^1-chromosome and *BRC/ABL* translocation, and could detect the *BCR/ABL* transcript in cases of Ph^1-positive essential thrombocythemia[29-31]. Ph^{1+} and *BCR/ABL*$^+$ ET and thrombocythemia associated CML

Table 11 Staging of JAK2^{V617F} positive prodromal polycythemia vera, erythrocythemic polycythemia vera, classical polycythemia vera, early myelofibrosis, inapparent polycythemia vera, spent phase polycythemia vera and post-polycythemia vera myelofibrosis according to 2015 World Health Organization-Clinical Molecular and Pathological criteria related to therapy

PV: WHO-ECMP stage	0	1	2	3	4	5	6
WHO-ECMP	Prodromal	Erythrocythemic PV	Early PV	Manifest PV	PV early MF	Inapparent	Spent PV
Clinical diagnosis	PV			Classical PV	Masked PV	PV	Post-PV MF
LAP-score	↑	↑	↑	↑	↑/↑↑	↑	Variable
EEC	+	+	+	+	+	+	+
Serum EPO	N/↓	N/↓	↓	↓	↓	↓	Variable
Erythrocytes × 10^{12}/L	> 5.8	< 5.8	> 5.8	> 5.8	> 5.8	Normal < 5.5	Decreased
Leukocytes × 10^{9}/L	< 12	< 12	< or > 12	< or > 15	> 15	N or ↑	> 20
Platelets × 10^{9}/L	> 400	400	< or > 400	> 400	< or >1000	N low or ↑	Variable
WHO-ECMP bone marrow	Early PV	Early PV	Early PV	Trilinear PV	Trilinear PV	Prilinear PV	Myelofibrosis
Bone marrow cellularity (%)	50-80	50-80	60-100	80-100	80-100	60-100	Decreased
Grading reticulin fibrosis: RF	RF 0-1	RF 0-1	RF 0-1	RF 0/1	RCF1/2/3	RCF 1/2/3	RCF 3/4
Grading myelofibrosis: MF[57]	MF 0	MF 0	MF 0	MF 0	MF 0/1	MF 0/2	MF 2/3
Splenomegaly on palpation	No/+	No	No/+	+	++/+++	++/+++	/large
Spleen size, echogram (cm)	< 12-15	< 13	12-15	12-16	18 > 20	16 > 20	> 20
Spleen size on palpation (cm)	0-3	NP	0-3	4-6	> 6	> 6	> 8
JAK2^{V617F} in granulocytes %	Low	Low	Moderate < 50	High > 50	High > 50	Mod/High	High > 50
JAK2^{V617F} in BFU-e (exon 12)	+(++)	+(++)	+(++)	++	++	+	++
Risk stratification → therapeutic implications anno 2014	Low risk	Low risk	Low risk	Intermediate risk PV	High risk PV early MF	Wait/see IFN JAK2	Post-PV MF Spent phase PV
First line Aspirin/Phlebotomy	Aspirin	Aspirin	Phlebotomy	Phlebotomy	If IFN resistant →	If IFN	JAK2
Second line IFN *vs* HU	Phlebotomy	Phlebotomy	Aspirin	Aspirin	HU or JAK2	Resistent	Inhibitor →
Third line JAK2 inhibitor			Low dose IFN → responsive	IFN → resistant → HU	inhibitor	JAK2 inhibitor	Bone marrow transplant

↑: Increased; ↓: Decreased; N: Normal; +: Present or heterozygous; ++: Homozygous; MF: Myelofibrosis; EPO: Erythropoetin; PV: Polycythemia vera; WHO-ECMP: World Health Organization European Clinical Molecular and Pathological; RF: Reticulin fibrosis; LAP: Leukocyte alkaline phosphatase; EEC: Endogenous erythroid colony; BFU-e: Burst forming units erythropoiesis; RCF: Reticulin collagen fibrosis; IFN: Interferon; HU: Hydroxy urea.

Table 12 2015 update on the molecular landscape findings in the chronic phase of essential thrombocythemia, polycythemia vera and myelofibrosis and during blast phase of myeloproliferative neoplasms transformation[121]

Gene	Chronic phase ET, PV and MF	Blast phase/AML
JAK2^{V617F}	PV: 95%-98%; ET and MF: 50%-60%	
MPL	ET: 1.5%; MF: 5%-10%	
TET2	PV: 7%-16%; ET: 4%-11%; MF: 8%-17%	
ASXL	PV: 2%; ET: 5%-8%; MF: 7%-17%	19%
DNMT3A	PV: 7%; ET: 3%; MF: 7%-15%	17%
CBL	MF: 6%	
LNK	PV, ET, MF: < 5%	10%
IDH 1/2	MF: 4%	21%
IKZF		19%
EZH2	MPNs: 5%-13%	
P53		27%
SRSF2		19%

MF: Myelofibrosis; PV: Polycythemia vera; ET: Essential thrombocythemia; AML: Acute myeloid leukemia.

vs the Ph^{1}- and *BCR/ABL*-negative thrombocythemias showed conspicuous differences in the form and size of megakaryocytes in bone marrow smears and sections of bone marrow biopsy. This difference of megakaryocyte histopathology observed in the Rotterdam cohort of CML and MPD patients described by Michiels *et al*[30] in 1987 appeared to be reproducible in bone marrow biopsies by the German pathologists Thiele *et al*[33,34] (1988, 1989) and Georgii *et al*[35,36] (1990, 1996) to distinguish between small mono or binucleated megakaryocytes as diagnostic for Ph^{1+} CML and ET *vs* large pleomorph megakaryocytes with hyperlobulated nuclei seen in the Ph-negative MPDs[37].

THE 1975 PVSG CRITERIA FOR ESSENTIAL THROMBOCYTHEMIA

PHT has already been defined in 1960 by Gunz[38] as clinical syndrome of recurrent spontaneous hemorrhages often preceded by thromboses, extremely high platelet count in excess of 1000 × 10^{9}/L, frequently splenomegaly, and hypochromic anemia with a tendency towards polycythemia between hemorrhages. Mucocutaneous bleeds from nose gums and gastrointestinal tract were most frequent followed by bruises and bleedings after trauma or surgery[38]. Accordingly, the PVSG used from 1975 to 1986 a minimum platelet count of 1000 × 10^{9}/L for the diagnosis for PHT without features of PV or AMM[8,38-40]. The PVSG inclusion and exclusion criteria in 1975 for the diagnosis of PHT or ET were very crude[8]: (1) A platelet count in excess of 1000 × 10^{9}/L and a bone marrow smear which shows marked megakaryocytic hyperplasia and abundant platelet clumps; (2) Absence of PV as defined by the PVSG normal red cell mass (RCM)[10]; (3) Absence of the

Philadelphia chromosome to exclude CML[16]; and (4) Absence of significant reticulin fibrosis (myelofibrosis) with dry tap on bone marrow aspiration, and no signs of preleukemia (erythroleukemia or trilinear myelodysplastic syndrome)[8,39,40].

PVSG defined PHT[8] labeled as ET[39,40] is featured by platelet counts between 1000 and 3000 × 10^9/L, splenomegaly in about 80%, autoinfarction of the spleen in 20%, and iron deficient microcytic anemia in 60%[39,40]. In 40% of PHT patients gastrointestinal roentgenograms suggest duodenal ulcer caused by small infarcts in the duodenal mucosa resulting from the high platelet count[40]. In the first prospective evaluation of PVSG defined PHT, 37 evaluable ET patients with platelet counts between 1000 to 2650 × 10^9/L suffered from thrombohemorrhagic events at presentation including mild bleedings in 5 epistaxis in 5, ecchymoses in 2, pelvic, buccal, fundal or urinary tract hemorrhage in 6, melena with a fall in hemoglobin of 7 gm/dL in 1 and massive postoperative bleeding in 1 case[39,40]. Eleven ET patients experienced acroparesthesias (numbness), including burning sensations, usually in hand or feet (suggestive for erythromelalgia), 9 had dizziness, light-headedness or syncope, 7 had visual disturbances such as scotomas and transient dimming or blurred vision. Catastrophic complications (severe hemorrhages, myocardial infarction, stroke) in 6 (16%) were observed. In this study of 37 untreated PHT or ET patients, bone marrow cellularity was normal in 11%, increased between 50% to 90% in 78% and greater than 90% in 11%[39,40]. Two-thirds of diagnostic bone marrow biopsies showed marked megakaryocyte hyperplasia with atypical large megakaryocytes. Reticulin content was essentially normal in 90% indicating prefibrotic MPD. MPD features in ET and PV were quiete similar: leukocytosis was common in PHT (ET) and PV, leukocyte alkaline phosphatase (LAP) scores over 100 were seen in 42% of PHT, and in 70% of PV patients; pruritis was observed in 14% in PHT and 43% in PV patients; the spleen was palpable in 38% of PHT and 70% of PV patients, and when enlarged in PHT the spleen was palpable 2 to 4 cm below the costal margin[8]. Since 1975 we discovered a causal relation between erythromelalgic microvascular disturbances and thrombocythemia in early early stage MPD disease at platelet counts above 400 × 10^9/L in symptomatic ET and PV patients with persistent increased platelet count in excess of 400 × 10^9/L and recognized that the increase of clustered large pleomorphic megakaryocytes in bone marrow biopsies was a pathognomonic clue to myeloproliferative thrombocythemia in ET and PV (Table 1).

IMPACT OF PVSG CRITERIA FOR PV ON DIAGNOSIS: RCM *VS* BONE MARROW HISTOLOGY

The PVSG established in 1975 clinical criteria (Table 2) for the diagnosis PV that are relatively simple to implement but rather crude thereby overlooking prodromal and masked PV when bone marrow histology as a pathognomonic clue to PV and ET is not considered or performed. The laboratory findings of 325 PV patients in the PVSG 01 study all PV patients had increased red cell mass by definition and showed an increase in hematocrit > 0.52 in 92%, white cells > 12 × 10^9/L in 43%, platelets > 400 × 10^9/L in 63%, LAP score > 100 in 70% and increased spleen size on palpation (splenomegaly due to myeloid metaplasia) in 70%[10]. Absence of splenomegaly was noted in about 30% of cases and leukocytosis and platelet counts can remain normal in early stage PV with typical PV bone marrow histology[10,41]. Such masked cases of PV with normal platelets, leukocytes and spleen were labeled in 1979 as idiopathic erythrocythemia (IE) by Pearson *et al*[42]. The 1975 PVSG criteria for PV in Table 2 exclude per definition IE (stage 1 PV, Table 11)[10,41]. IE is featured by increased red cell mass, normal spleen size, normal leukocyte and platelet counts and no clinical or laboratory evidence of primary or secondary erythrocytosis[42]. LAP scoring[8,17], *in vitro* cultures of erythroid progenitors (EEC)[43] and radioimmunoassay of erythropoetin (EPO) do contribute to differentiate PV from all variants erythrocytosis. These assays are useful when there is only isolated elevation of the red cell mass and all the usual causes of secondary polycythemia have been excluded. The characteristic histology findings in bone marrow biopsies of 155 evaluable PV patients with a documented increased RCM in the PVSG 01 study revealed a broad spectrum of bone marrow cellularity from 50% to 60% in 10 cases, from 60% to 80% in 45 cases, and from 80% to 100% in 100 cases (Figure 4)[41]. Silver stained reticulin fiber content was normal (RF-0 and 1 = prefibrotic) in 94 cases, slightly increased (RF-2 = early fibrotic) in 40 cases, and moderately to marked increased (RF-3) in 21 cases. The bone marrow histology diagnoses in the PVSG-01 study[41] could roughly be interpreted as typical for normocellular ET in 10, for PV (hypercellular 60%-80%) in 45, for trilinear PV in 70 and for PV/RF-3 or 4 in 13 PV patients (Figure 4). The bone marrow histology data of megakaryopoiesis in PVSG PV and PHT studies were identical in appearance, and the condition PV *vs* ET could not be distinguished on megakaryocyte histology[39-41]. Increased bone marrow cellularity due to increased erythropoiesis and/or myelopoesis in PVSG defined PV and PHT or ET were identical. The PVSG concluded that the condition PV *vs* ET could not be distinguished on the basis of bone marrow histopathology. Consequently, the PVSG only used increase RCM and did not include bone marrow histology as the determinative major inclusion criterion for the diagnosis of PV, and to separate ET from PV[10,39-41]. RCM is insensitive and not specific for the diagnostic differentiation of PV, IE, SE and inapparent PV (Table 2)[17]. In contrast, EEC and bone marrow histology are specific clues for the diagnosis of PV since the early 1970s[1-3,41-43].

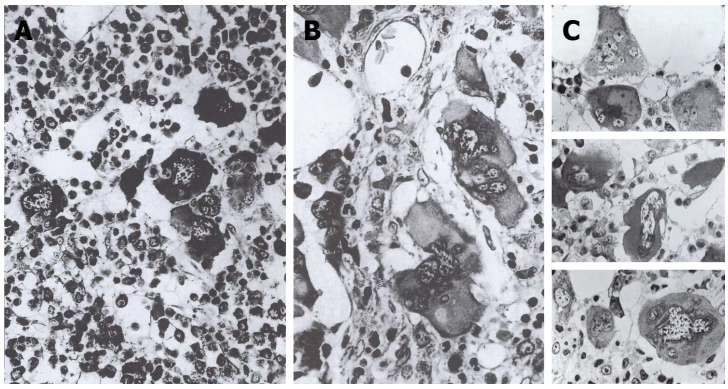


Figure 4 Cluster of medium and large immature megakaryocytes (350 × left) and large megakaryocytes with immature cytoplasm and immature clumpsy cloudlike nuclei (pseudolobulation) (right 900 ×) and very slight reticulin fibrosis in chronic megakaryocytic granulocytic myeloproliferation, and characteristics of megakaryocytes in prefibrotic myeloproliferative disorders: myeloproliferaive disease, 900 × plastic embedding. A: Deep hyperlobulated nuclei in essential thrombocythemia; B: Pleomorp large megakaryocytes in Polycythemia Vera; C: Small and large and immature cloud-like nuclei in chronic megakaryocytic granulocytic myelosis or primary megakaryocytic granulocytic myeloproliferatio). Georgii *et al*[35,36].

From 1988 to 1994, Lamy *et al*[44] measured RCM in 103 consecutive patients seen in a single center diagnosed in 85 cases (83%) as PVSG defined PV patients with increased hemoglobin (Hb) and hematocrit (Ht) defined, respectively, by Hb > 18 g/dL, Ht > 0.52 in males and Hb > 16 g/dL, Ht > 0.47 in females and as inapparent PV (IPV) in 18 patients (17%) (Table 3)[44]. IPV was defined by a normal Hb and Ht value at diagnosis. In the IPV group, the reasons to perform RCM were as follows: splenomegaly associated with increased platelets and/or leucocytes counts (n = 8), portal vein thrombosis (n = 5), increased platelets or leukocytes counts without splenomegaly (n = 3), and isolated splenomegaly (n = 2)[44]. The two groups were balanced in terms of age, sex, leukocyte, serum iron, and platelet level. Hemoglobin, Ht levels, red cell counts, and plasma volumes were significantly different between the two groups (Table 3). Red cell mass was increased in the two groups due to hypervolumemia in PV, but caused by splenomegaly in cases with IPV, whereas the erythrocyte counts were increased in the majority of 85 PV patients, but completely normal in 18 IPV patients except one (Table 3). Consequently IPV with normal or decreased hemoglobin, hematocrit and erythrocytes in Table 3 cannot become candidates for phlebotomy because of absence of hypervolemic symptoms. Treatment will hydroxyurea carry the great danger of inducing relative anemia and acceleration of myelofibrosis. In the context of splanchnic vein thrombosis (portal or splenic vein thrombosis), masked and overt Budd Chiari syndrome RCM and blood volume are increased due to splenomegaly as the cause of IPV[45]. We observed in 1990 a case with post-PV myelofibrosis and gigantosplenomegaly with increased RCM, 37 mL/kg, increased plasma volume (hypersplenism) at a hemoglobin level of 2.1 mmol/L (6.0 g/dL) erythrocytes 2.9 × 10^{12}/L and platelet count of 35 × 10^{9}/L. After splenectomy (spleen weight 5000 g) resulted in correction of the hemoglobin to 7.8 mmol/L (12.5 g/dL) and a postsplenectomy hemorrhagic thrombocythemia with platelet counts of 3000 to 4000 × 10^{9}/L associated with the paradoxical occurences of platelet medidiated erythromelalgia and spontaneous hemorrhages (bruises, nose bleedings) caused by severe acquired von Willerand disease type 2A VWF: Ag 10.8 U/mL VWF:RCo 0.48 U/mL, VWF:CB 0.27U/mL with the absence of large and intermediate VWF multimer. Such occult and overt cases of splanchnic vein thromboses secondary to hepatic, portal or splenic vein thrombosis is related to splenomegaly and hypersplenism keeping the blood counts of platelets and erythrocytes normal or decreased inducing masked stage PV with typical EEC and bone marrow histology[2,17,41-44]. In our analysis in 1997 of the series by De Stefano *et al*[45] 13 out of 33 patients with splanchnic vein thrombosis had spontaneous EEC and could be diagnosed according to classic PVSG criteria as PV in 5, ET in 1, MF in 1 and masked primary MPD in 6[45]. The high frequencies of gastric ulcers and gastritis estimated at 20% to 30% in patients with Budd-Chiari syndrome or splanchnic vein thrombosis result in iron deficiency accompanied by hypochromia and microcytosis, which do explain a substantial additional decrease in hemoglobin levels as compared to still normal erythrocyte counts as could be observed in the study of Lamy on IPV (masked PV). On behave of the European Working Group on Myeloproliferative Disorders (EWG.MPD) Briere and Michiels introduced in the late 1990s bone marrow histology as a pathognomonc clue to MPD in patients with splanchnic vein thrombosis (SVT)[45,46]. In a single-center retrospective study of 128 patients with SVT, clusters of abnormal megakaryocytes in bone marrow biopsy combine with EEC were used as reference standard for the diagnosis of MPD (including PV, ET and masked MPD)[46]. In the group of 129 SVT patients 31 had definitive MPD positive for both BMB and EEC, 63 had no MPD with a negative result of BMB and EEC and 34 were positive for either BMB or EEC[46]. Kiladjian *et al*[47] assessed the diagnostic and prognostic value of JAK2 and MPL^{515} mutations in 241 SVT patients

(104 BCS, 137 PVT). JAK2^{V617F} was found in 45% of BCS and 34% of PVT, while JAK2 exon 12 and MPL^{515} mutations were not detected[47]. JAK2^{V617F} was found in 96.5% of patients with BM changes specific for MPD and EEC, but also in 58% of those with one feature (BM or EEC), and in 7% of those with neither feature indicating the superiority of JAK2 screening for detection of MPD in SVT patients[47]. In the meta-analysis of Smalberg *et al*[48], JAK2^{V617F} screening in SVT patients without typical WHO defined MPN features identified masked MPN disease in 17.1% and 15.4% who usually did have typical features of MPN on bone marrow histology evaluation[48].

IMPACT OF PVSG CRITERIA FOR PV ON TREATMENT

In the PVSG 01 study of 431 PV patients randomized for phlebotomy in 134, chlorambucil in 141 and P^{32} in 156, there was a significant loss of survival of PV patients due to major thrombotic complications during the first 3 years in the phlebotomy arm due to uncontrolled thrombocythemia and aiming at a too high haematocrit just below 0.50[9,10,49,50]. In retrospect this would not be the case with the recommendation of phlebotomy aiming at a haematocrit of 0.40 according to Dameshek and Pearson *et al*[42] on top of aspirin in the United Kingdom and The Netherlands since 1985[51]. There was a striking increased incidence of overall malignant complications in PV patients with P^{32} and chorambucil as compared to the phlebotomy-treated PV patients during long-term treatment[49,50]. The overall incidence of leukemia/lymphoma and cancer after 10 to 11 years follow-up was 25% in the phlebotomy arm, 40% in the P32 arm and 67% in the clorambucil arm. The increased incidence of malignancies of bone marrow, lymphoid tissue, skin, and gastrointestinal tract highlights mutagenic effects of chronic myelosuppressive agents in particular when treatment is already started in newly diagnosed early and overt stages of PV. The PVSG 01 trial confirmed the hypothsis of Dameshek[3,50,51] that P^{32} is leukemogenic when used as the first line myelosuppressive treatment in early and overt stage PV indicating the need to postpone myelosuppressive therapy in PV as long as possible[52-55]. A large group of low risk PV patients included in the PVSG 01 study were exposed to the leukemogenic agents P^{32} and chlorambucil. The PVSG 01 investigators recommended around 1990 to replace P^{32} by hydroxyurea as the first treatment option in PV patients[52-55]. The final analysis of the French PVSG study compared hydroxyurea (HU) (n = 136) *vs* pipobroman (n = 149) as first-line therapy in 285 newly diagnosed PV patients younger than 65 years (27 patients were older than 65 years)[56]. During follow-up 42 patients (31%) switched from HU to pipobroman because of HU toxicity and 19 (13%) from pipobroman to HU[56]. According to the intention to treat, the median survival was 17 years for the whole cohort, 20.3 years for the HU arm, and 15.4 for the pipobroman arm (P = 0.008). At 10, 15 and 20 years, the cumulative incidence (probability) of AML/MDS was 6.6%, 16.5% and 24% in the HU arm *vs* 13%, 34% and 52% in the pipobroman arm. The cumulative incidence (probability) of MF at 10, 15 and 20 years was 15%, 24% and 32% in the HU arm *vs* 5%, 10% and 21% in the pipobroman arm (P = 0.02)[56]. Results from PV patients who received only one treatment during the entire period (HU n = 94, Pipobroman n = 130) the cumulative incidence of AML/MDS at 10, 15 and 20 years was 7.3%, 10.7% and 16.6% for HU *vs* 14.6%, 34% and 49.4% for pipobroman[56].

The baseline risk of leukemic transformation in 459 PV and 605 ET patients treated in a single institution retrospective study without cytoreductive therapy or hydroxyurea alone was 3.3% and 7.4%, respectively[57,58]. A primary rigid venesection regimen according to the London PV study group in the late 1970[59-61] aiming at a hematocrit around to below 0.40 irrespective of gender on top of low dose aspirin introduced by Michiels *et al*[51] in 1985[62-64] will reduce the cumulative incidence of minor circulatory ischemic events and major thrombosis from above 50% to less than 2% per patient/year during long-term follow-up[59-64]. Hydroxyurea has to be postponed in early and intermediate stage PV (Table 11) as long as possible by phlebotomy on top of low dose aspirin[17,65]. Current risk stratification in PV and ET should not anymore be based on age above 60 years and history of thrombosis[55], but on real life MPN disease burden using objective parameters including the degree of leucocytosis, splenomegaly, JAK2 allele burden, itching and constitutional symptoms[65]. HU and JAK2 inhibitors are indicated according to not yet clearly defined recommendations in hyperproliferative PV with increased to high MPN disease burden (Table 11) althought targeted recommendations on the use of JAK2 inhibitors will be further clarified and defined soon[66].

THE 1990 HANNOVER BONE CLASSIFICATION OF THE PH-NEGATIVE MPD

In 1987 Georgii and Michiels discussed their experiences that bone marrow histology features of Ph-positive CML *vs* the Ph-negative MPDs reflect distinct disease entities simple because the differences in megakaryocyte size and morphology is so obvious that cytologists and pathologists can easily distinguish the small monolobulated megakaryocytes in Ph-positive CML and ET from the large pleomorphic megakaryocytes in the Ph-negative MPDs ET and PV[14,17,67,68]. The PVSG distinguished four distinct categories of myeloproliferative disorders (CML, ET, PV and AMM or PMF) and a fifth category of unclassifiable MPD. The concept of this traditional classification by the PVSG and the WHO in 1979 has been revised by Georgii *et al*[35,36] in his Hannover Bone Marrow classification of the MPDs as

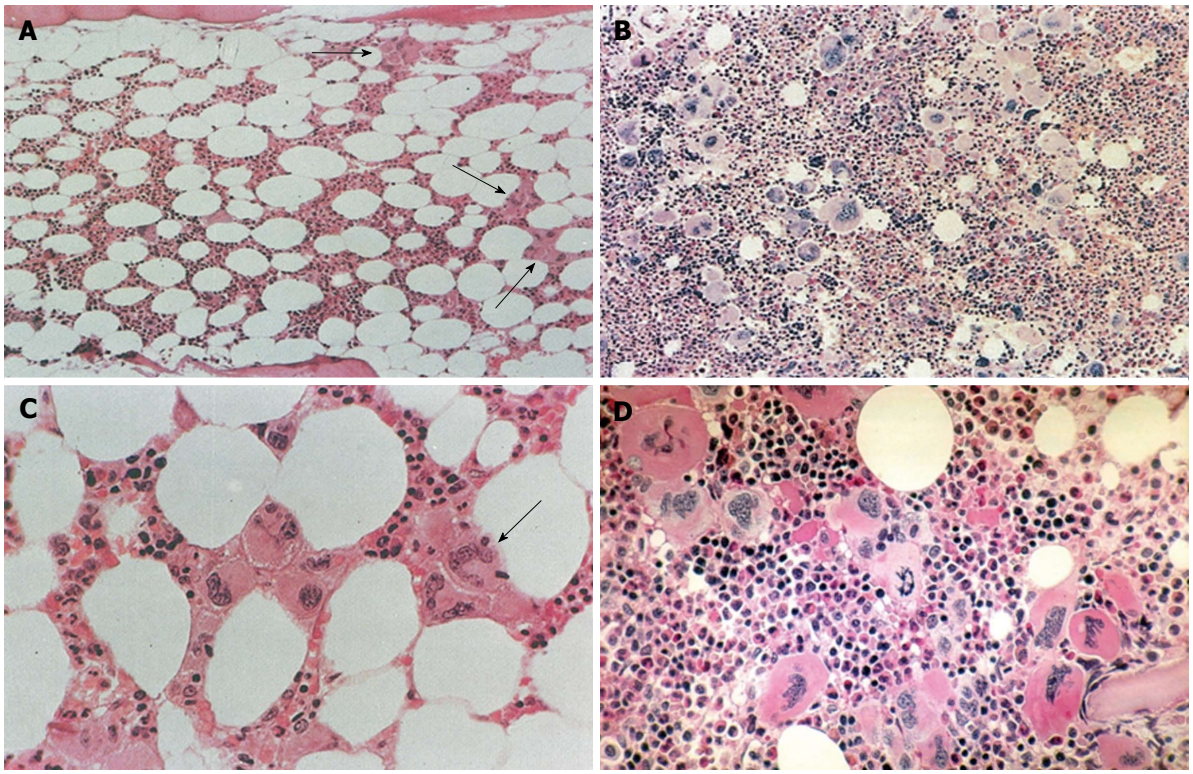


Figure 5 Spectrum of essential thrombocythemia and polycythemia vera bone marrow features in 155 bone marrow biopsies with clustered large pleomorphic megakaryocytes in polycythemia vera patients from the polycythemia vera study group 01 study. ET bone marrow histology (A and C): arrows indicate clustered pleiomorphic megakaryocytes. PV (B) with trilinear megakaryo/eryhtro/granulocytic hyperplasia. Source Ellis *et al*[41] polycythemia vera study group 1975 and Wasserman *et al*[49]. An ET picture was observed in 10 PV. An ET/PV bone marrow picture with increased cellularity (60%-80%, D) was detected in 45 PV. A hypercellular (80%-100%, B) PV picture was recorded in 90 bone marrow biopsies of 155 PV patients the PVSG 01 study. PV: Polycythemia vera; ET: Essential thrombocythemia.

follows: primary diseases are CML, PV, thrombocythemia, chronic megakaryocytic granulocytic myeloproliferation (CMGM) and unclassifiable MPD[35-37]. Reticulin and collagen fibrosis is a reactive feature consecutive to myeloproliferation[35]. Georgii *et al*[35] distinguished three bone marrow histology types of *BCR/ABL*-positive CML: CML of common type with a predominance of granulopoiesis (CML.CT), CML with megakaryocyte increase (CML.MI), and CML with megakaryocytes predominance (CML.MP)[35]. The blood and bone marrow presentation of BCR/ABL-positive ET without features of CML is similar as has been described for CML with megakaryocyte predominance (CML.MP)[30-32,37]. The reliable distinction within the three Ph-negative MPDs and its variations appears to be problematic caused by an overlapping cytomorphology of megakaryocyte and within the three MPDs thrombocythemia, PV and CMGM According to Georgii and Michiels an MPD classification system should be focused cytomorphology of megakaryocytes from bone marrow smears and histopathology from bone marrow biopsies[30-32,35,36]. Megakaryocyte morphology and bone marrow histology had become a hallmark of distinction for the diagnostic differentiation of thrombocythemia (ET), PV *vs* CMGM in the Hannover Institute of Pathology since 1980[67,68]. For the understanding of MPD classification bone marrow histology should distinguish between primary prefibrotic and advanced diseases[35,36]. Primary prefibrotic MPDs according to the Hannover Bone Marrow Classification in 1990 include PV, normocellular thrombocythemia and hypercellular thrombocythemia associated with CMGM without any feature of PV (Figure 5)[35,36]. Results from Adamson *et al*[69], Fialkow *et al*[70,71] and many others revealed the reactive nature of reticulin and collagen fibrosis within the MPDs[69-74]. Consequently, the term of chronic idiopathic myelofibrosis (CIMF) or PMF can not be considered as correct in the Hannover Bone Classification of the MPDs since it has to be considered as a consequence of an underlying myeloproliferative disease[35-37,73,74]. In the 1990 Hannover bone marrow classification, Georgii *et al*[35,36] omitted the terms AMM, CIMF and PMF and introduced the entity of CMGM as the third distinct entity without features of PV and ET. The diagnosis of prefibrotic CMGM is mainly based on the presence of large immature megakaryocytes with immature cytoplasm and cloud-like nuclei not seen in ET and PV (Figure 5)[35,36]. The term CMGM has illogically been changed into CIMF under the influence of Thiele and Vardiman in the 2001 WHO classification[75] and as PMF in the 2008 WHO classification by Tefferi and Thiele[76]. With the advent JAK2 and MPL mutations as driver causes of clonal ET and PV Michiels *et al*[77,78] recognized CMGM as the third distinct MPD entity and replaced the term CMGM by JAK2/MPL wild type primary

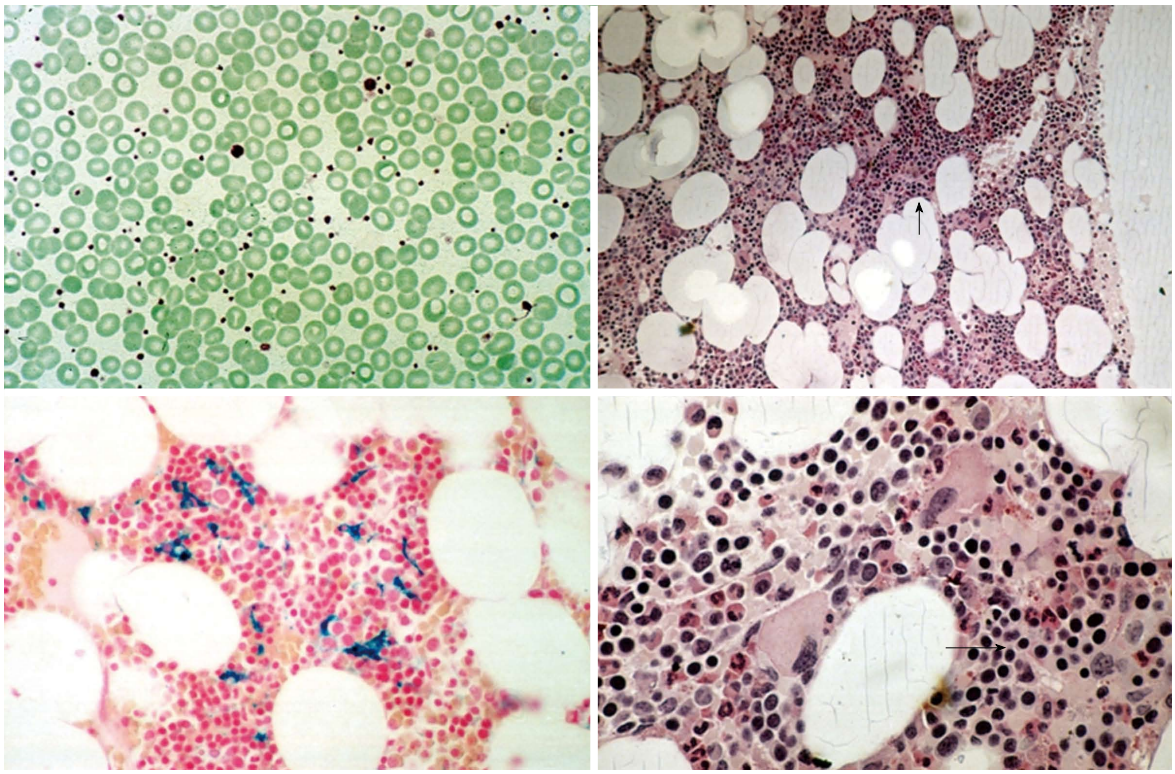


Figure 6 Presence of large platelets in peripheral blood smear (left top), increase of clustered enlarged megakaryocytes in a normocelluar essential thrombocythemia bone marrow with stainable iron (left bottom). Local increase of erythropiesis (long arrow) in areas of loose clustered pleiomorphic megakaryoctyes in patients with normocellular essential thrombocythemia: essential thrombocythemia bone marrow histology.

megakaryocytic granulocytic myeloproliferation (PMGM) in the 2015 WHO-CMP classification (Table 11)[77,78].

THE 1980 ROTTERDAM CLINICAL AND PATHOLOGICAL CRITERIA FOR ET AND PV

Since 1976 the German pathologists Georgii[79] and Burkhardt *et al*[80] had clearly defined the pathological features of ET, PV and CMGM[67-80] on the basis of which we could developed the Rotterdam clinical and pathological (RCP, Table 1) criteria for ET and PV[81,82]. As the 1975 PVSG criteria for ET (PTH) are crude, we could recognize since 1975 the existence of erythromelalgic thrombotic thrombocythemia (ETT) in early stage MPD with a persistent increase of platelet count in excess of 400 × 10^9/L[17,81,82]. The trephine biopsy in these cases showed a proliferation of large mature megakaryocytes in a normal cellular bone marrow with normal erythropoiesis and granulopoiesis (Table 1 and Figure 6). At that time we followed since 1975 the definition of PV according to Dameshek[2] as a trilinear MPD[3]. In PV increased erythropoiesis was most prominent[76] together with variable degrees of increased platelets (> 400 × 10^9/L)[10], erythrocytes (> 6 × 10^{12}/L)[2,3] and granulocytes in the peripheral blood in the absence of the Ph-chromosome (Table 1) and could document distinct bone marrow features as the pathognomonic clue to very early stage of ET (Figure 6). The 1980 RCP criteria of ET and PV were determined by careful prospective documentation of peripheral blood and bone marrow smears and bone marrow biopsy material (Table 1). Platelets in excess of 400 × 10^9/L, and an increase of clustered enlarged megakaryocytes in a bone marrow biopsy material was found to be pathognomonic diagnostic for ET and excluded reactive thrombocytosis. The combination of bone marrow histology and erythrocyte count above 6 × 10^{12}/L according to Dameshek[1,2] appeared to be specific clues to the diagnosis of PV clearly different from all variant of primary and secondary erythrocytosis as documented by Kurnick *et al*[83] in 1972 and by Vykoupil *et al*[67] in 1980. The 1980 RCP modifications of the 1975 PVSG criteria for PV include 4 main changes (Table 1). First, the major criterion O_2-saturation of > 92% is replaced by absence of primary or secondary erythrocytosis by clinical and laboratory tests. Second; splenomegaly is replaced by bone marrow histology as a major criterion (A3). Third, the 1980 RCP diagnostic set used splenomegaly as a minor criterion (Table 1). Fourth, we skipped raised B12 (> 900 ng/L) or raised B12 binding capacity (> 2200 ng/L) as completely irrelevant for the diagnosis of early and overt stage PV (Table 1).

Between 1975 and 1980, we prospectively evaluated the RCP criteria in 30 consecutive early prefibrotic stage patients, who presented with ETT, 14 ET and 16 PV patients[84]. The mean age of 30 ETT patients (ET and PV)

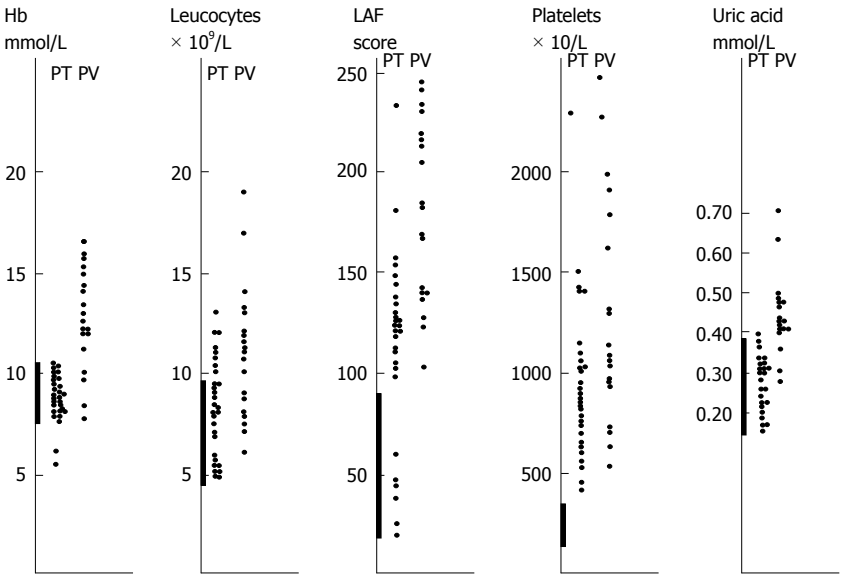


Figure 7 Laboratory findings at time of presentation in symptomatic with thrombocythemia subdivided in 30 primary thrombocythemia (= essential thrombocythemia) and 20 polycythemia vera patients seen between 1975 and 1985. Hb levels are normal in PT and elevated in PV[16]. Leukocytes were normal or elevated in PT and PV. Leukocyte alkaline phosphatase score was much more elevated in PV than in PT with normal values in 6 PT patients. Platelet counts were were between 400 and 1000 × 10/L in the majority of the 30 PT patients. Serum uric acid levels were normal in PT and usually elevated in PV. PT: Primary thrombocythemia; LAP: Leukocyte alkaline phosphatase; PV: Polycythemia vera.

was 56.7 (range 33-96) years. Eleven of 14 ET patients had platelet counts below 1000 × 10^9/L, in whom the diagnosis of ET would have been overlooked by the crude PVSG criteria at that time between 1975-1985. Spleen size on scan was slightly increased in 5 of 14 ET and in 13 of 16 PV patients. Leukocyte count counts was increased (> 10 × 10^9/L) in 5 out of 14 patients with ET and in 14 of 16 PV patients. LAP score was increased (> 100) in 12 out of 14 ET and in all PV patients. All PV patients with erythrocytes above 6 × 10^{12}/L had increased red cell mass (manuscript in preparation). Increased erythrocyte counts above 6 × 10^{12}/L and increase of large pleomorphic megakaryocytes in bone marrow smear (Dameshek)[2,3] and biopsy is diagnostic for PV[17]. Erythrocyte count at a cutoff level of 6 × 10/^{12}L differentiates ET from PV on top of a typical MPD bone marrow histology obviating the need to measure RCM (Table 1). Increased erythrocytes above 6 × 10^{12}/L persists in PV in a comple hematological remission by repeated venesection[2,3,77,78].

The presence of clustered large pleomorph megakaryocytes in bone marrow smears and biopsies is diagnostic clue for ET and PV (Table 1, Figures 4 and 6). An ET bone marrow picture with increase of clustered pleomorphic megakaryocytes and no increase of cellularity (Figure 5) was seen in 7 of 14 ET and only in 1 of 16 PV patients[83,84]. A moderate increase of cellularity (60%-80%) in the bone marrow due to increased erythropoiesis (= decreased M/E ratio) consistent with early stage PV was seen in 3 ET and 4 PV patients. A typical PV hypercellular (80%-100%) bone marrow due to megakaryo/erythro/granulocytopoiesis (panmyelosis according to Dameshek[1]) was seen in one ET patients and in the majority of PV[83,84]. These results indicate that bone marrow histopathology on its own is characteristic for MPN but not fully reliable to differentiate between ET and PV (Figures 4 and 6)[77,78]. The peripheral blood findings in 30 ET [primary thrombocythemia (PT)] and 20 PV seen between 1975 and 1985 are shown in Figure 7[84]. Hemoglobin levels are normal in ET and elevated in PV, leukocytes were normal or elevated in ET and PV[84]. Out of 30 ET patients 24 had an increased and 6 had a normal LAP scores (Figure 7). Platelet counts were were between 400 × 10^9/L and 1000 × 10^9/L in the majority of the 30 ET patients. Serum uric acid levels were normal in ET and frequently elevated in PV. Increased LAP score in the absence of infection and normal erythrocyte sedimentation rate (ESR) or C-reactive protein (CRP) is indicative for MPN PV or ET (erf). LAP score was much more elevated in all 20 PV than in ET[84]. As leukocyte alkaline phosphatase (LAP) scoring is becoming increasingly rare in common practice, it can easily be replaced by CD11b neutrophil expression[85]. In the setting of SVT patients PV patients showed higher CD11b values of 190 (CI: 151-238) in PV *vs* 111 (CI: 81-153) in non-PV patients with SVT[85]. In routine practice CD11b expression in the MPNs PV, ET of various molecular etiology *vs* all variants of erythrocytosis should be assessed aganist CD11b expression in controls who have normal ESR and CRP in order to kick out false positive due to underlying infectious or systemic diseases.

THE COLOGNE CLINICAL AND PATHOLOGICAL CRITERIA FOR PH^{1-} NEGATIVE MPD

In the 1980s Thiele *et al*[86,87] described two variants of ET. The histopathology of "true" ET as compared

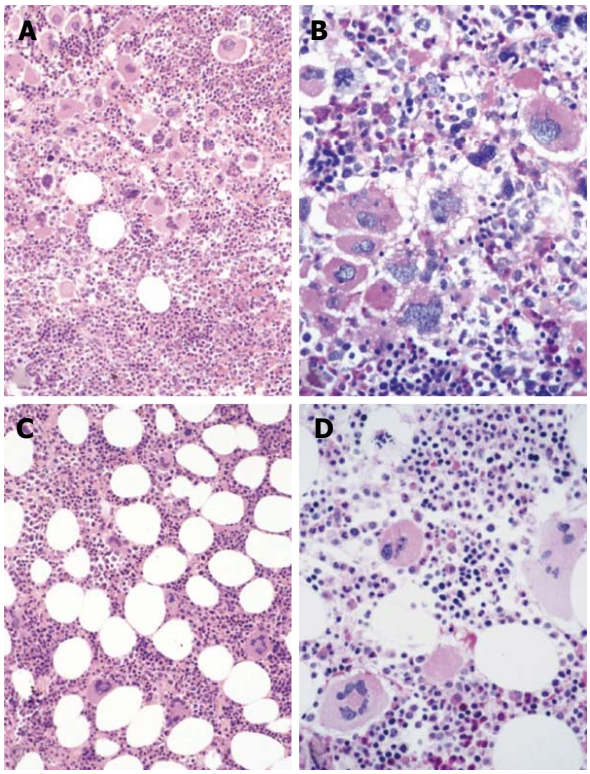


Figure 8 Hypercellular false and normocellular true essential thrombocythemia. A and B: Hypercellular false ET or prefibrotic PMF according to Thiele (Table 6) consistent with CMGM according to Georgii *et al*[35] (1990), or primary megakaryocytic granulocytic myeloproliferation (PMGM122) featured by dense clustered immature megakaryopoiesis and the predominance of immature cloud-like nuclei (Kvasnicka), which are not seen in JAK2V617F positive ET and PV; C and D: Normocellular True ET (World Health Organization-ET, Table 5) according to Thiele and Michiels 1988-2006[59,62,107] with the presence of large to giant megakaryocytes larger than in JAK2^{V617F} positive ET and PV. True ET as defined by Thiele seems to our experience most consistent with normocellular JAK2 wild type ET carrying the MPL^{515} mutation featured by clustered large and giant megakaryocytes in a normocellular bone marrow, which usually runs a very benign course. ET: Essential thrombocythemia; PV: Polycythemia vera; CMGM: Chronic megakaryocytic granulocytic myeloproliferation.

to PV in bone marrow biopsy is characterized by uniform appearance of mature large and giant mature megakaryocytes with hyperlobulated nuclei (staghorn) in a normal cellular bone marrow dispersed among normal granulopiesis and erythropoiesis (Figure 8)[86-88]. At the bone marrow level classical PV was featured by a marked pleomorphism of the megakaryocyt (small, medium and large megakaryocytes) in ET and PV in combination with an increased erythropoiesis with or without increased granulopiesis, whereas the megakaryocytes were of normal size in controls and reactive thrombocytosis[86-90].

In 1999 Thiele *et al*[90] introduced the Cologne Clinical and Pathological (CCP) criteria and defined the bone marrow features of normocellular "true" ET, hypercellular trilinear PV, and hypercellular ET associated with prefibrotic chronic idiopathic myelofibrosis (false ET or prefibrotic CIMF). The CCP criteria of thrombocythemias in various MPDs were based on a retrospective clinico-pathological study of 395 PVSG defined MPD patients with platelet count above 500 × 10^9/L (Table 4)[81]. For comparison 35 patients with reactive thrombocytosis were enrolled in this study. The 395 MPD patients PV (*n* = 55), CIMF = PMF (*n* = 250 prefibrotic and fibrotic), prefibrotic PMF (*n* = 120) and "true" ET according to the CCP criteria (Tables 3 and 4). In 2002 Michiels and Thiele defined "true" ET and differentiated "true" ET from ET associated with prefibrotic CIMF (Table 5, Figure 8)[82]. In "true" ET megakaryocytes display large to giant megakaryocytes showing hyperlobulated staghorn-like nuclei in a normocellular bone marrow (Figure 8)[78,80]. PV was typically featured by large pleomorphic megakaryocytes with hyperploid nuclei in a hypercellular bone marrow due to increased erythropoiesis or increased erythrocytic-megakaryocytic-granulocytic myeloproliferation. Interestingly the megakaryocytes in "true" ET were larger than in PV (Table 5, Figure 8)[1]. Hypercellular ET associated with prefibrotic CIMF ("false" ET = CMGM = PMGM, Figure 7) is dominated by an increase of clustered atypical dysmorphic megakaryocytes due to increases of cellular and nuclear size and bulky nuclei with clumsy lobuli and irregular roundish shaped form (so-called cloud-like nuclei, Figures 7 and 8), which are never described in ET and PV[2]. Normocellular "true" ET according to the 2002 European clinical pathological (ECP) criteria is featured by normal LAP scores (normal CD11b neutrophil expression), higher platelet counts and large to giant megakaryocytes with multilobulated stag-horn like nuclei in a completely normocellular bone marrow (Table 5)[90,91]. In "true" ET the values for hemoglobin, erythrocytes, and LAP scores (CD11b neutrophil expression) were completely normal (Table 5). In contrast, the RCP defined prefibrotic normocellular ET and prodromal PV were associated with increased LAP score (Figure 7), the presence of pleomorphic megakaryocytes (Figure 6) low serum EPO levels, slight splenomegaly, and spontaneous EEC[17,84]. This point to the existance of at least two phenotypes of normocelluar ET: ET with features of early PV (prodromal PV) *vs* "true" ET without features of PV. These differences in RCP defined ET (Table 1) criteria and CCP defined "true" ET and false ET (Table 3) are related to a selection bias of patients. In 1988, Thiele as a pathologist selected 25 cases with 1975 PVSG defined normocellular ET (minimum platelet count of 1000 × 10^9/L) who had pronounced thrombocythemia and normocellular bone marrow without PV features and this was associated with normal LAP score (CD11b neutrophil expression)[87]. Focussing on aspirin-sensitive erythromelalgic inflammatory and ischemic complications as pathognomonic presenting symptoms of early ET or PV patients, the Rotterdam MPD Working group studied a selected and biased group of early stage myeloproliferative ET and PV at platelet counts above 400 × 10^9/L[84]. Only symptomatic ET and PV patients were included in our 1975-1985 studies because of erythromelalgic ischemic digital circulation disturbances[84].

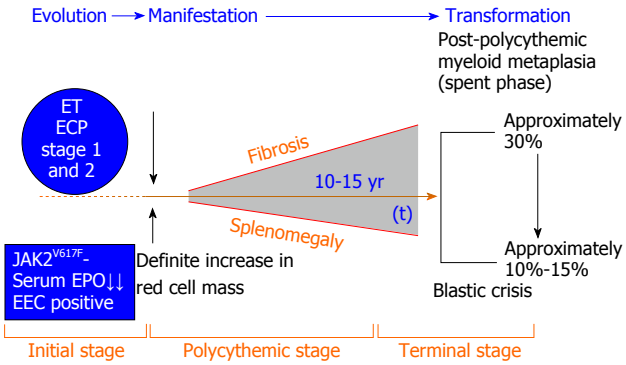


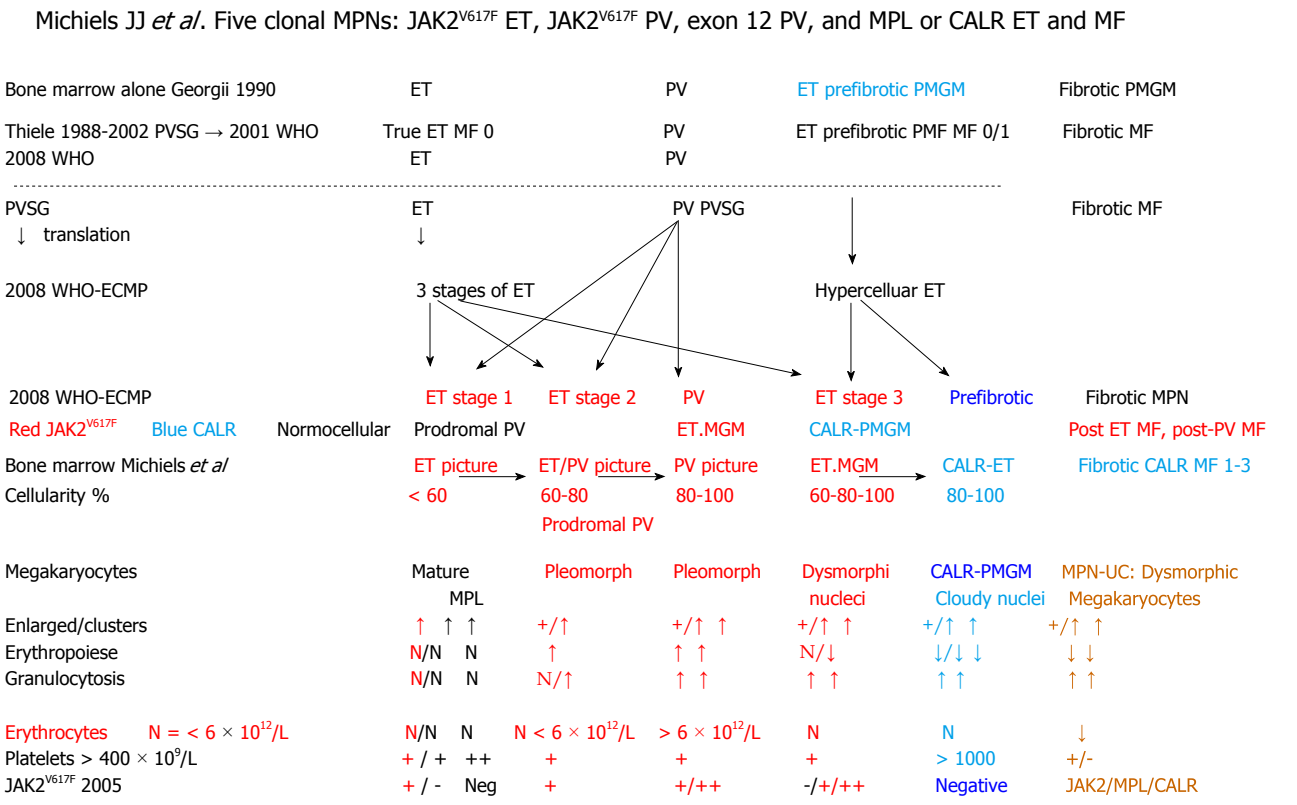
Figure 9 Prodromal polycythemia vera with normal red cell mass (red cell mass, Tables 3 and 7) and erythrocytes (< 6 × 10^{12}/L, Tables 8 and 9), prefibrotic idiopathic erythrocythemia and polycythemia vera with increased erythrocytes (> 6 × 10^{12}/L) and red cell mass, and evolution into masked PV with splenoegaly, spent phase polycythemia vera with myelofibrosis (Table 12) and post-polycythemia vera-myelofibrosis according to European Clinical and Pathological criteria defined by Michiels and Thiele (http://www.mpn-stichting.nl/doctors_brochure_2004.pdf) and according to the 2006-2007 World Health Organization-European Clinical Molecular and Pathological criteria for JAK2 mutated stages of prodromal overt and advanced polycythemia vera[71-73].

THE 2006-2008 EUROPEAN CLINICAL, MOLECULAR AND PATHOLOGY CRITERIA FOR JAK2^{V617F} MUTATED AND JAK2 WILD TYPE MPN

Myelofibrosis is not specific for a disease and can be observed in patient with hairy cell leukemia, Ph-positive CML and in the Ph-negative MPDs, CMGM, PV, ET and many other conditions as well[35-37,92,93]. An increase of reticulin in the silver impregnation stain of bone marrow biopsies can be graded as fine or coarse fibers and is a secondary event relevant for prognosis assessment[94]. The use of CIMF and PMF in the 2001 and 2008 WHO classifications[75,76] is misleading and scientifically not recommended[94]. We persisted to use this term in the 2006/2007 WHO-ECMP classification[94-96] for reasons of compliance with peer reviewers, but we convincely eliminated the terms CIMF and PMF in the 2013-2014 WHO-CMP classifications (Tables 7-11) for the MPNs of JAK2^{V617F} positive ET, PV, JAK2-negative ET and hypercellular ET associated with PMGM[77,78]. MF itself is the reflection of MPN disease progression but not a disease entity on its own because reticulin and collagen fibrosis are produced by polyclonal fibroblasts in response to cytokines released from the clonal granulocytic and megakaryocytic proliferative cells in both PV and ET of various molecular etiology[69-74]. The presence of reticuline fibrosis is well documented in all variants of ET, PV, PMGM, CML and in many other conditions. An increase of reticulin fibrosis is rare in WHO normocellular ET will occur in about one third of PV and will occur in the majority of patients with PMGM during long-term follow-up[35,36,89].

The WHO bone marrow features and the variable phenotypes of thrombocythemias according to the 2002 ECP (Tables 5 and 6) and 2006/2007 ECMP criteria for ET (http://www.mpn-stichting.nl/doctors_brochure_2004.pdf) (Tables 6, 7 and 8) do in fact distinguish within the JAK2^{V617F} mutated MPN normocellular (WHO-ET, < 60%) and hypercellular ET (prodromal PV, 60%-90%) due to increased erythropoiesis, from JAK2 wild type normocellular ET and separate JAK2 mutated MPN from JAK2 wild type hypercellular ET associated with a granulocytic myeloproliferation (PMGM, Figures 9 and 10)[94-96]. JAK2^{V617F} mutated normocellular ET and prodromal PV patients had increased LAP score similar as in PV (Figure 7). Bone marrow histopathology on its own was not reliable to differentiate between JAK2^{V617F} positive ET, prodromal PV and classical PV (Table 6), whereas the 2002 ECP criteria had defined "true" ET with normal LAP score and giant megakaryocytes with staghorn-like nuclei in a normocellular bone marrow (Table 5) and prefirotic CIMF = PMF = PMGM (table) very likely belonging to the JAK2 wild type MPNs (Table 5 and Figure 10)[91]. According to Michiels *et al*[91], WHO bone marrow biopsy histopathology evaluations have a sensitivity and specificity near to 100% to differentiate all variants of ET in various MPD/MPNs from thrombocythemia in CML, from myelodysplastic syndromes and from reactive thrombocytosis[94-96]. The distinction of prefibrotic thrombocythemias in the MPNs of various molecular etiology at the bone marrow level is a topic of research for the Dutch and Belgian hematopathologists[77,78]. The ECP, ECMP and WHO bone marrow features separate myeloproliferative PV from all variants of primary congenital erythrocytoses and secondary erythrocytoses[77,78]. Bone marrow histology separates idiopathic erythrocytosis with increased RCM from early erythrocythemic stage 1 PV, and do detect "masked" ET, "masked" PV overlooked by the PVSG and WHO criteria with a sensitivity and specificity of 100% if the trephine biopsy is of good quality (Table 6 and Figure 10)[77,78]. A bone marrow biopsy is mandatory for grading cellularity in prefibrotic stages and for grading reticulin and collagen fibrosis (Table 1) in hypercellular MPN advanced PV and ET (post-ET myelofibrosis, post-PV myelofibrosis, Figure 9).

In 2007 Thiele left the European Working Group on MPD and joined again the WHO investigators to define the 2008 WHO classification of the myeloproliferative neoplasms ET, PV and PMF[76,97,98]. The formulation of the WHO-ECMP criteria in 2006/2007 by Michiels *et al*[94-96] (Tables 7 and 8, Figure 11) for ET prodromal PV and PV preceded the publication of the 2007/2008 revision of the WHO classification[96-98]. Thiele *et al*[99] validated the 2008 WHO classification of MPN in JAK2^{V617F} positive and JAK2 wild type MPN from large series of PVSG defined MPD patients diagnosed in the past and persisted to use the terms "true" ET *vs* "false" ET or PMF[100-102]. The 2008 WHO investigators were persistently confronted with unsolved problems and pitfalls regarding in ET of various MPNs and did not recognize the distinct differences in bone marrow histology between JAK2 mutated ET and PV and JAK2 wild type ET without features



Conclusion: for clarity the WHO-ECMP MPN classification and staging separate JAK2^{V617F} positive classical trilinear PV and ET stage 1, 2 and 3 (in red) *vs* JAK2 wild type (blue) ET stage 3 = PMGM, and fibrotic endstage of PMF.

Figure 10 Charaterization of polycythemia vera study group defined essential thrombocythemia and polycythemia vera by applying the 2006 European Clinical Molecular and Pathological criteria and 2001 World Health Organization bone marrow features; JAK2^{V617F} mutated normocellular essential thrombocythemia, prodromal polycythemia vera, classical polycythemia vera and myelofibrosis *vs* JAK2 wild type normocelluar essential thrombocythemia and chronic idiopathic myelofibrosis or Primary megakaryocytic granulocytic myeloproliferation. WHO: World Health Organization; PV: Polycythemia vera; ECMP: European clinical molecular and pathological; MF: Myelofibrosis; ET: Essential thrombocythemia; MPN: Myeloproliferative neoplasm; PMGM: Primary megakaryocytic granulocytic myeloproliferation; PVSG: Polycythemia vera study group; CALR: Calreticulin.

of PV. The 2008 WHO recognized the differences in megakaryocyte morphology in CML, MDS as compared to PV but did not pick up the potential importance of significant differences of megakaryocyte morphology between JAK2, MPL and CALR mutated ET and MF[78]. The 2015 WHO-CMP classification of the prefibrotic MPNs distuinguishes JAK2^{V617F} mutated trilinear ET and PV (Tables 7-9, Figure 11) from exon 12 PV and based on distinct megakaryocyte morphology features to differentiates JAK2^{V617F} mutated ET and MF from JAK2 wild type "true" ET carrying the MPL mutation (Table 10) and from JAK2/MPL wild type hypercelluar ET carrying the calreticulin (CALR) mutation associated with PMGM (Table 11, Figure 8).

2015 WHO-CMP CLASSIFICATION OF FIVE DISTINCT CLONAL MPNS

JAK2^{V617F} mutated trilinear PV and ET: From Dameshek to Vainchenker

The one cause hypothesis of trilinear PV proposed by Dameshek[2] in 1950[3] has been confirmed by Vainchenker in 2005 (Figure 1) by the discovery of the JAK2^{V617F} mutation as the driver cause of ET, PV, masked PV and MF[103,104]. Detection of JAK2^{V617F} has become the first intention diagnostic test to differentiate between PV and myeloproliferative IE from erythrocytosis with a sensitity of 95% and specificity of 100%[105-116]. The prevalence of the JAK2^{V617F} mutation in PVSG defined PV is 95% and about 50% in ET and MF[7,105-107]. The JAK2^{V617F} mutation load is low in ET and ranges from less than 10% to 50% of the granulocytes, either low less than 50% or high between 50% to 100% (homozygous) of the granulocytes positive for the heteroxygous/homozygous JAK2^{V617F} mutation in PV (Figure 12)[110-112]. A group of JAK2^{V617F} positive normocellular ET with a very low percentage of heterozygous mutant JAK2 can maintain as a non-progessive subpopulation in the bone marrow without a tendency to evolve into prodromal PV or hypercellular ET during long term follow-up[115]. The few patients with hypercellular ET homozygous for the JAK2^{V617F} mutation patients are at high risk for anemia on one hand and myeloid metaplasia of the spleen (splenomegaly) with secondary myelofibrosis on the other hand (Figure 9)[112]. The percentage of JAK2^{V617F} positive granulocytes in PV may range from rather low to 100% for JAK2^{V617F} during the long-term follow-up. Hetero/homozygous or homozygous JAK2^{V617F} mutation is associated with pronounced constitutively activation of megakaryopoiesis, erythropiesis and granulopoiesis in the bone marrow as the cause of hypercellular trili-

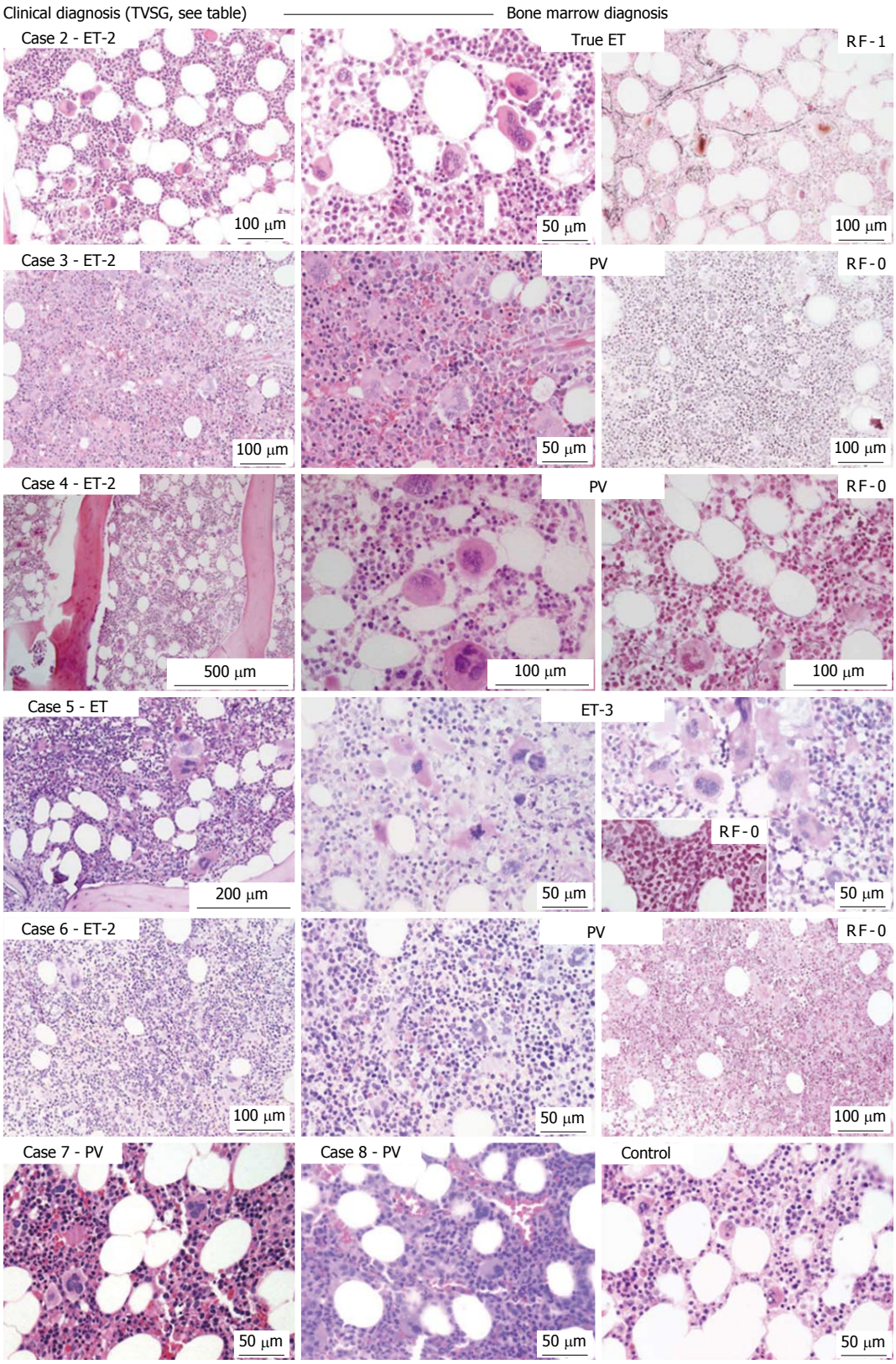


Figure 11 The clinical features of four JAK2^{V617F+} essential thrombocythemia and two JAK2^{V617F+} polycythemia vera cases refer to the numbering of cases in Figure 10. Case 2: Clinically JAK2^{V617F} ET 2 (low serum erythropoetin) and a normocellular ET [World Health Organization (WHO)-ET] bone marrow wit pleomorph small and large megakaryocytes and reticulin fibers (RF) grade 1; Case 3 and 4: Clinically JAK2^{V617F} ET 2 with a trilinear hypercellular PV bone marrow and RF 0 in case 3: ET 2 with increased cellularity due to increased erythropoiesis RF grade 0 in case 4; Case 5: Clinically JAK2^{V617F} ET with moderate splenomegaly and a hypercellular megakaryocytic granulocytic myeloproliferation (ET.MGM = ET 3), with dysmorphic megakaryocytes (not cloud-like) and RF grade 0 in case 5; Case 6: Clinically JAK2^{V617F} ET 2 with a trilinear PV bone marrow picture1 and RF grade 0; Case 7: Clinically JAK2^{V617F} PV with a 65% hypercellular ET/PV bone marrow picture in between "normocellular ET" (WHO-ET) and trilinear hypercellular (90%-100%) PV picture in case 7 and 8 with PV with increased RCM and erythrocytes above 6 × 10^{12}/L. In 2007, we concluded that bone histology alone does not differentiate between JAK2 mutated ET and PV, as compared to control, morphology of pleiomorph megakaryocytes in JAK2^{V617F} mutated ET and PV are similar Source Poster P-0025. Fourth International Congress on myeloproliferaive disease/ myelodysplatic syndrome New York, 2007. ET: Essential thrombocythemia; PV: Polycythemia vera.

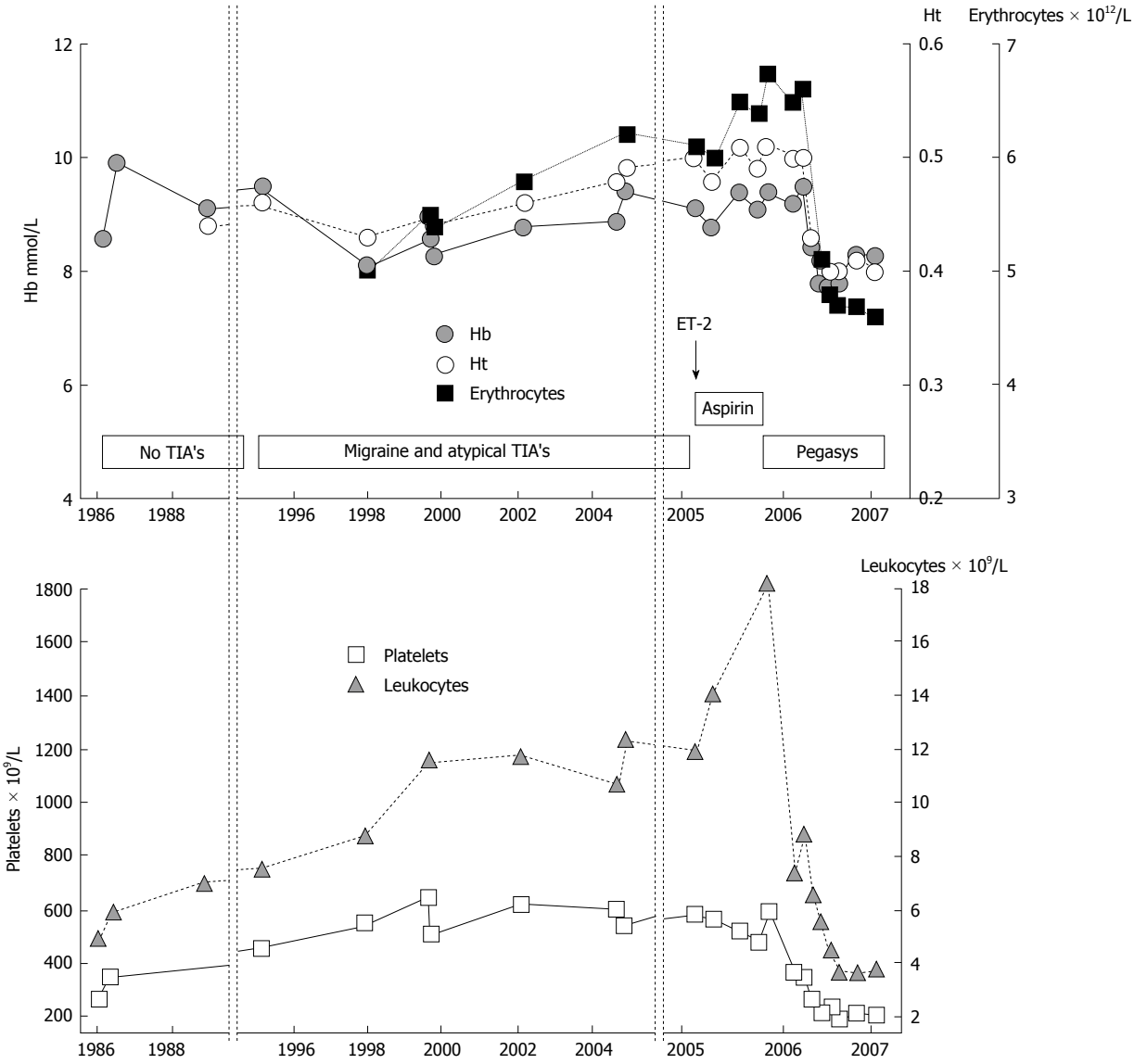


Figure 12 Sequential stages of essential thrombocythemia 1995-2004, prodromal polycythemia vera 2004-2005 and overt polycythemia vera 2006, with a complete hematological response (complete hematological responses, case 6, Table 9, Figure 11) and subsequent complete hematological responses of interferon for 6 years at age of 72 years anno 2013.

near PV[2,3,103,104]. Scott *et al*[113] and Moliterno *et al*[114] demonstrated that so-called heterozygous PV with allele load less than 50% are hetero/homozygous at the EEC level in blood and bone marrow for the JAK2^{V617F} mutation, whereas ET patients are heterozygous reflecting a maximal JAK2^{V617F} mutation load of 50%[113,114]. Heterozygous JAK2^{V617F} mutation leading to constitutively activated megakaryocytes with increased sensitivity to TPO is enough to induce ET and to produce constitutively activated, hypersensitive, sticky platelets responsive to aspirin (aspirin-responsive Sticky Platelet Syndrome)[116,117]. Godfrey *et al*[118] studied the JAK2 mutation status of burst forming unit-erythropiesis grown in low erythropoietin conditions in 77 patients with PV or ET[113]. Using microsatellite PCR to map loss-of-heterozygosity breakpoints within individual colonies, homozygous mutant colonies were absent or present in low percentages in heterozygous ET, but prevalent and common in patients with JAK2^{V617F}-positive PV[118]. In this study of Godfrey *et al*[118], PV has been distinguished from ET by expansion of one dominant homozygous subclone, the selective advantage of which is likely to reflect additional cytogenetic[119], genetic or epigenetic alterations (Table 12)[120-123]. Such additional, acquired background biological factors on top of the JAK2, MPL and CALR driver mutations of MPN will become of huge importance for the understanding of differences in prognosis and outcome.

Prospective evaluation of WHO-CMP criteria in JAK2^{V617F} mutated MPN

Bone marrow cellularity, increased erythropoiesis or granulopoisis and the morphology of pleomorphic megakarocytes are not different in JAK2^{V617F} mutated ET and PV (Figure 11). Normocellular ET had stable ET disease without any progression during life long follow-

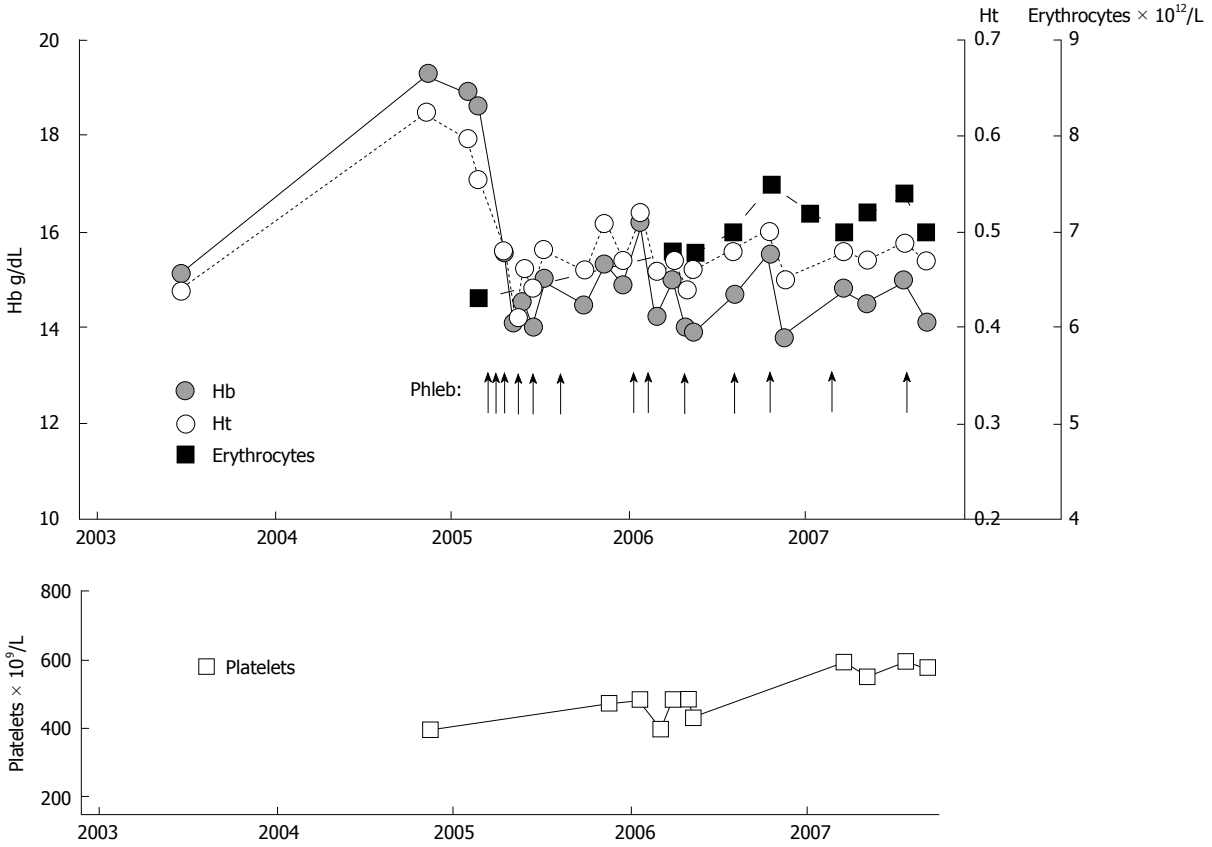


Figure 13 Clinical course in case 8 (Table 9, Figure 11) with erythrocythemic polycythemia vera treated with venesections (arrows). The development of microcytic hypochromic erythrocytes due to iron deficiency was associated with persistent increased red cell count (> 6 × 10^{12}/L), which is diagnostic for polycythemia vera. The iron deficient state and the low normal values for haemoglobin (Hb) and hematocrit (Ht) was associated with relief of hypervolumic symptoms with phlebotomy on top of low dose aspirin.

up (Figures 13 and 14). As shown in Figure 13 the sequential evolution of ET, prodromal PV and overt PV is predicted to response to pegylated inteferon (Pegasys^{R}) treatment. PVSG defined ET patients frequently had a typical hypercellular PV bone marrow picture due to increased erythropoiesis (prodromal PV, cases 3, 4, 5 in Figure 11) similar as observed in newly diagnosed PV patients (cases 7, and 8 in Figure 11). As shown in Figure 14 stage 1 JAK2^{V617F} mutated pure erythrocythemia or idiopathic erythrocytosis according to PVSG criteria presented with a typical PV bone marrow histology (case 8, Figure 11) and persistant increased erythrocyte counts above 6 × 10^{12}/L. After correction of haemoglobin and hematocrits to to around 0.40 by repeated venasections (Figure 14) the erythrocyte counts remained above 6 × 10^{12}/L whereas the JAK^{V617F} mutation load increased in this case raised from heterozygous 25% to homozygous 65% after 5 years follow-up.

Detection of JAK2^{V617F} mutation and serum EPO measurement have become the first step in the diagnostic work-up of erythrocytosis with erythrocyte counts above the upper limit of normal (> 5, 6 × 10^{12}/L)[124-126]. Vannucchi *et al*[111] employed quantitative assays for JAK2^{V617F} allele levels in granulocytes in a prospective study of 175 PV patients at time of diagnosis[111]. The JAK2 mutant allele burden could be quantified as 1%-25%, 25% to 50%, 50%-75% and 75%-100% in 57, 50, 34 and 32 PV patients respectively at time of investigation[111]. The burden of JAK2^{V617F} allele was directly correlated with abnormally increased levels of hematocrit, white cell and neutrophil count, LDH and LAP score, spleen size on echogram and with decreased values for serum ferritin, and erythropoietin[111]. The JAK2^{V617F} mutation load in this study nicely correlated with a progressively higher relative risk for aquagenic pruritus, spleen size on echogram, total thrombosis and the need for receiving myelosuppressive.

Among patients with SVT (Budd-Chiari syndrome or portal vein thrombosis) are MPN in an early phase, the so called "masked" ET or PV which not yet meet the 2008 WHO criteria[47,48]. In the Kiladjian *et al*[47] study, 241 of such SVT patients of whom 74 had platelet counts between 238 and 456 × 10^{9}/L (mean 333) who carried the JAK2^{V617F} mutation. In 147 SVT patients JAK2 wild type masked MPN the platelet count varied between 104 and 258 × 10^{9}/L (mean 159)[47]. Interestingly, none of the 241 SVT cases carried the MPL^{515} mutation, whereas congenital factors for venous thrombosis like Factor V Leiden were rather frequent[47]. In the meta-analysis of Smalberg *et al*[48] the JAK2^{V617F} mutation was identified in 276 of 1002 (28.5%) patients with Budd-Chiari syndrome and in 173 of 855 (19.4%) patients with portal vein thrombosis. A total of 268 SVT patients were tested for JAK2 exon 12 and 305 for

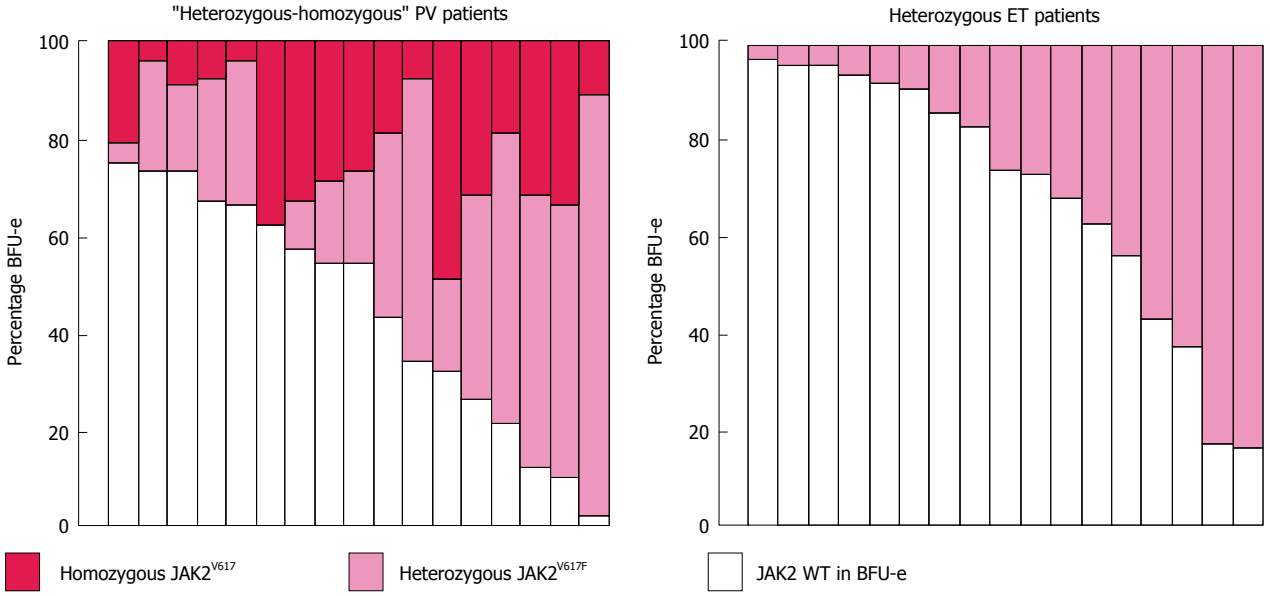


Figure 14 Genotype of individual BFU-E in polycythemia vera and essential thrombocythemia with granulocytes heterozygous for the JAK2^{V617F} mutation (less than 50% JAK2^{V617F} mutation load) show that polycythemia vera patients are heterozygous/homozygous and essential thrombocythemia patients heterozygous for the JAK2^{V617F} somatic mutation. ET: Essential thrombocythemia; PV: Polycythemia vera; WT: Wild type; BFU-e: Burst forming units erythropoiesis.

MPL^{515} mutations. Three patients were found to carry the MPL^{515} mutation and JAK2 exon mutation was not detected in any of these SVT patents[48]. Three studies concluded that JAK2^{V617F} mutation can be considered pathognomonic for MPN in patients with splanchnic vein thrombosis[47,48,127]. These SVT patients with portal hypertension, splenomegaly and hypersplenism suppress and consequently mask the elevated blood cell counts, which can be expect in active MPN disease[47,48,127]. Masked cases of early stage ET and PV in patients with splanchnic vein thrombosis are overlooked by the 2008 WHO classification[128] and nowadays easily detected by combining JAK2^{V617F} mutation screening and WHO-CMP criteria[77,78]. The 2015 WHO-CMP classification recognizes the existence a broader spectrum of JAK2 mutated normocellular ET, prodromal PV, IE, early PV, overt PV, masked PV (not meeting the WHO criteria), advanced PV with splenomegaly and post-PV myelofibrosis, which will has significant therapeutic implications (Table 11)[77,78].

JAK2 exon 12 mutations as cause of idiopathic erythrocythemia and PV

The frequency of JAK2 exon 12 mutations among all patients with PV is estimated around 3%[129,130]. JAK2 N542-E543del is the most frequent among the different reported exon 12 mutations. The finding of the JAK2 exon 12 mutations in the 5% PV patients negative for JAK2^{V617F} usually present with early stage PV or idiopathic erthrocytosis (IE = increased red cell mass with normal values for leukocytes and platelets and no palpable spleen) with a favourable outcome and normal life expectancy[129-131]. Ten JAK2 exon 12 mutated MPN patients had increased red cell mass, were negative for the JAK2^{V617F} mutation, and could be diagnosed as PV in 6 and idiopathic erythrocytosis in 4[129]. Pre-treatment bone marrow histology in JAK2 exon 12 mutated PV or IE showed characteristic erythroid hyperplasia with minor and distinct histology changes of the megakaryocyte lineage, which are never seen in primary or secondary erythrocytoses[129]. Cases of exon 12 mutated, JAK2^{V617F} negative PV were frequently diagnosed as IE with increased hematocrit hematcrit and red cell mass, low serum EPO, normal platelet and leukocyte counts, no or palpable spleen and a typical hypercellular bone histopathology predominantly due to erythroid hyperplasia and clusters of large megakaryocytes with hyperploid nuclei[129-131]. In the bone marrow histology study of Lakey *et al*[131] in 7 cases of JAK2 exon 12 mutaed PV all showed prominent erythroid hyperplasia meeting the criteria for IE in 4 and PV in 2, but hyperplasia of atypical small to medium-sized large megakaryocytes was present in all (Figure 15)[131]. Presenting features at diagnosis were aquagenic pruritis and/or erythromelalgia in 3, and microvascular events including headache, dizziness, blurred vision and distal extremity numbness in 4 at platelet counts between 152 and 790 × 10^9/L (5 below and 2 above 400 × 10^9/L consistent with aspirin responsive platelet thrombophilia[116] and the sticky platelet syndrome (SPS)[117]. Six of 7 JAK2 exon 12 MPN cases were diagnosed as IE with low serum EPO levels in all. Bone marrow pathology of the JAK2 exon 12 PV cases lacked the prominent clustering of large megakaryocytes with hyperlobulated nuclei that characterize JAK2^{V617F} mutated PV[77,78]. Bone marrow pathlogy in JAK2 exon 12 mutated MPN revealed a spectrum of small to medium sized megakaryocyte with a predominance of

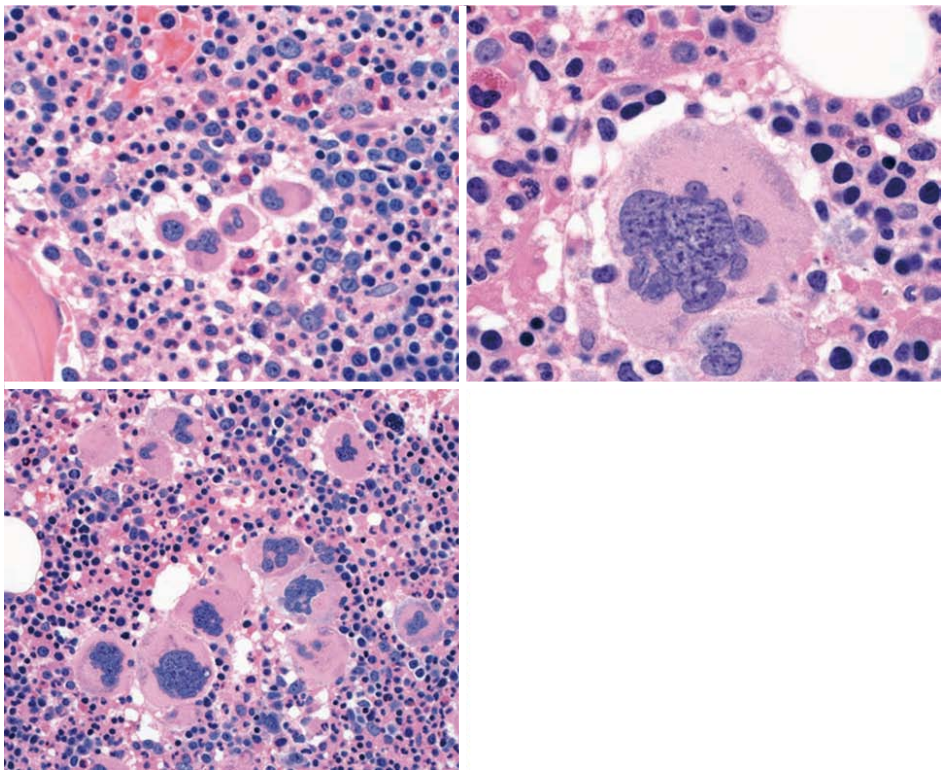


Figure 15 Bone marrow histology in JAK2 exon 12 polycythemia vera.

smaller forms. The nuclei were atypical with a varying degree of lobation comprising monolobulated and hyperlobulated forms. The chromatin was abnormally distributed (Figure 15)[131]. The bone marrow reticulin content was normal or slightly increased in 6, and one case evolved 15 years after intitial diagnosis into post-PV myelofibrosis with reticulin fibrosis grade 3 and associated osteomyelofibrosis.

Hereditary autosomal dominat essential thrombocythemia due to a gain of function mutation in the TPO and JAK2 gene

In the 1990s, studies on murine leukemia and oncogenes led to the recognition of a new member of the hematopoietin receptor super family. It was discovered as the product of the gene c-mpl, the normal cellular homologue of the oncogene v-mpl, the transforming principle of a murine myeloproliferative leukemia virus, responsible for a panmyeloid transformation[130]. This was followed by the molecular cloning and characterisation of Mpl, the human homologue of the c- and v-mpl[132-135]. The receptor MPL was then rapidly recognized as being the thrombopoietin receptor (TpoR) by the demonstration that antisense oligonucleotides of c-mpl inhibited the colony-forming of megakaryocyte progenitors by Wendling *et al*[136]. The Mpl ligand became the key to the identification of TPO and was cloned in 1994 by five independent groups[136-140]. The Mpl ligand is identical to thrombopoietin and labeled as megakaryocyte growth and development factor MGDF[139-142]. Human TPO has all the functions ascribed to MGDF, and all MGDF-like activity can be neutralized by soluble recombinant Mpl[143]. TPO stimulates hematopoietic stem cells and megakaryocyte precursors to proliferate, differentiate and mature and mature megakaryocytes form pro-platelets, which then disintegrate into platelets[143].

Two basic research studies clearly showed that continuous forced expression of TPO, (TPO^{high} mice) in mice induces megakaryocyte proliferation and differentiation and subsequently develop myelofibrosis[144-146]. TPO^{high} mice engineered to overexpress TPO in their liver and those that received transplants of marrow cells infected with a TPO containing retrovirus develop thrombocythemia due to massive hyperplasia of megakaryocytes and granulocytes and hypoplasia of erythropoiesis in the bone marrow followed by myelofibrosis and extramedullary hematopoiesis within 2 to 3 mo and die from myeloid metaplasia and myelofibrosis thereafter[144]. TGF-Beta-1 has been implicated in the pathobiology of myelofibrosis by the observation that megakaryocytes from TPO^{high} rats and mice express high levels of TGF-Beta-1 in marrow extracellular fluids and plasma[145]. In wild mice TGF-Beta-1 mRNA expression in bone marrow and spleen was barely detectable before TPO treatment, and significantly increased in both organs after TPO treatment and returned to basal levels at day 14 (Figure 9)[145]. Another growth factor produced by megakaryocytes, platelet derived growth factor, was found to be upregulated in a fashion similar to TGF-Beta-1. High levels of TGF-Beta-1 mRNA in bone marrow and spleen cells in TPO^{high} mice were associated with high levels of TGF-beta-1 protein in extracellular fluids from

these organs.

The first well documented report on autosomal dominant hereditary ET (HET) due to a gain of function mutation in the *TPO* gene was described in a large Dutch family[147-149] and in a Polish family[150]. HET due to gain of function mutation in the *TPO* gene is associated with marked increased TPO levels (530 ± 27 pg/mL *vs* controls < 62 pg/mL), and microvascular circulation disturbances including erythromelalgia and atypical transient ischemic attacks consistent with aspirin responsive platelet thrombophilia[116] and the sticky platelet syndrome[117]. Increase of large platelets and large mature megakaryocytes with hyperploid nuclei and normal cellularity in bone marrow biopsy specimens of the propositus (man born in 1934) and affected family members were diagnostic for HET, which was associated with platelet-mediated thrombophilia comparable as first discovered by Michiels *et al*[51,81,82] in 1985 and as described by Vannucchi *et al*[111] in acquired JAK2^{V617F} mutated ET and PV[110,111].

Hereditary autosomal dominant ET caused by a gain of function mutation in the *JAK2* gene R564Q and V617I produces a typical WHO-ET pictures of blood and bone marrow without features of PV[151-153] and do present with typical manifestations of the SPS[116,117]. Etheridge *et al*[151] and Mead *et al*[152] described a novel germline mutation JAK2^{V617I} in a family with autosomal dominant HET. Peripheral blood and bone marrow histology are consistent with WHO-ECMP defined ET (Figure 11)[151,152]. The authors demonstrated that JAK2^{V617I} is the sole driver in JAK2^{V617I}-positive individuals with typical peripheral blood and bone marrow features of WHO normocellular ET and completely normal values for haemoglobin, haematocrit, erythrocytes plasma TPO and serum EPO. After stimulation with granulocyte colony-stimulating factor (G-CSF), TPO and EPO of peripheral blood CD33+ myeloid and CD34^{+}stem and progenitor cells showed significant differences in congenital JAK2^{V617I} and acquired JAK^{V617F} mutated cells as compared to controls. The response to G-CSF was increased in congenital JAK^{V617I} and more prominent in acquired JAK2^{V617F} mutated HSC. In signalling and transcriptional experiments assays, congenital JAK2^{V617I} showed more activity than wild type acquired JAK2, but substantially less than JAK2^{V617F}. The responses to TPO were equal in congenital JAK2^{V617I} and acquired JAK2^{V617F} but the response to EPO was normal in congenital JAK^{V617I} and increased in acquired JAK2^{V617F}. These findings confirm the hypothesis of Vainchenker that heterozygous congenital JAK2^{V617I} mutation induces sufficient cytokine hyperresponsiveness of the HSC to TPO for the induction of a homogeneous ET phenotype in blood and bone marrow without features of PV during long term follow-up[152].

A novel heterozygous JAK2^{R564Q} mutation has been identified in another family with autosomal dominant HET[153]. The growth promoting effects of congenital JAK2^{R564Q} were much milder than those of acquired JAK2^{V617F} mutation. The authors found higher levels of STAT1 and STAT3 in cells expressing JAK2^{V617F}, compared to JAK2^{R564Q}. Total STAT1 levels were increased with JAK2^{V617F} and with JAK2^{R564Q} expression as compared to wild type JAK2 but this effect was more prominent with the somatic acquired JAK2^{V617F} mutation. An overall increase in downstream signaling in mutant JAK2^{R534Q} cells was further demonstrated by the upregulated tyrosine-phosphorylation of proteins in germline JAK2^{R564Q}-expressing cells as compared to wild type JAK2, and this was even more robust in the acquired JAK2^{V617F}-expressing somatic mutants. Similar increased signaling was observed in JAK2^{R564Q}-positive patients by the demonstration that increased phosphorylation of JAK2 protein in platelets isolated from 3 members of the family with the congenital JAK2^{R564Q} mutation as compared to a JAK2 wild type family members[153]. In the absence of TPO, and at all concentrations of TPO, the growth characteristics of congenital JAK2^{R564Q}-expressing cells showed significantly increased proliferation, compared to JAK2 wild type cells, but this was much less striking than with acquired JAK2^{V617F} mutated cells thereby explaining why the heterozygous germline JAK2^{R564Q} mutation is associated with ET without PV features[153].

JAK2 wild type MPL^{515} mutated ET

The first case of congenital ET due to a gain of function mutation in the *cMPL* gene has been described in 2004[154]. This has led in 2006 to the discovery of the MPL^{W515L} and MPL^{W515K} mutations as the driver cause of clonal MPN in JAK2 wild type ET and myelofibrosis by Pardanani *et al*[155] and Pikman *et al*[156] in the United States[149,150]. Three studies describe MPL^{W515L} and MPL^{W515K} mutations as the cause of clonal ET and myelofibrosis without features of PV (Table 9)[155,156]. Within the JAK2 wild type MPN, the prevalence of the MPL^{5155} mutation as the cause of ET (Table 9) is 3% in the Vannucchi study[157], and 8.5% in the United Kingdom studies[157,158]. In the study of Vannucchi *et al*[157], patients with JAK2 wild type ET carrying the MPL^{515} mutation present with typical Sticky Platelet Syndrome[116,117] but have no clinical, laboratory and bone marrow features of prodromal PV at diagnosis, do not evolve into overt PV during follow-up, have normal serum EPO, normal ferritin levels, absence of spontaneous EEC[157,158]. The bone marrow is featured by pronounced megakaryopoiesis with large and giant megakarocytes and no increase of erythropoiesis[156-159]. In 2008 we studied bone marrow histopathology in 12 cases with JAK2 wild type ET carrying the MPL^{515} mutation kindly provided by the courtesy of Dr. Vannucchi, Florence, Italy[78]. Bone marrow histology from patients with JAK2 wild type ET carrying the MPL^{515} mutation consistently displayed clusters small and large megakaryocytes with a greater number of giant megakaryocytes with hyperlobulated stag-horn nuclei in a normal cellular bone marrow and no increase of erythropoiesis (Table 10). As compared to JAK2^{V617F} mutated ET in Figure 16, bone marrow histology of our case with MPL^{515} mutated ET is shown in Figure 17. The comparison of bone marrow histopathology findings in patients with normocellular

JAK2^{V617F} mutated ET (Figures 11 and 16) *vs* JAK2 wild type ET carrying the MPL^{515} mutation (Figure 17) show were significant differences on three points[78]. The megakaryocytes in MPL^{515} mutated ET are larger with hyperlobulated staghorn-like nuclei as compared to the pleomorphic megakaryocytes morphology in JAK2^{V617F} mutated ET and PV. Second, there was local increase of erythropoiesis in areas of loose clustered pleiomorphic megakaryocytes in JAK2^{V617F} mutated ET, but not in JAK2 wild type PT carrying the MPL^{515} mutation (compare Figures 16 and 17). Third, we observed increased reticulin fibers grade 2 in a normocellular bone marrow in areas of dense clustered megakaryocytes, which is not seen in JAK2^{V617F} mutated normocellular ET, hypercellular prodromal PV and EMGM[77]. Whether such differences in bone marrow histology and megakaryocyte morphology between normocellular ET with low JAK2 mutation load and normocellular JAK2 wild type ET carrying the MPL^{515} mutation can be seen by expert hematopathologists in large series of WHO-ET patients remains to be evaluated in prospective clinical and basic research studies.

PMF or AMM: From Silverstein to Tefferi

In 1975 the PVSG defined the criteria for PV and for both primary hemorrhagic thrombocythemia and PMF or AMM as the second and third variant of MPD[8]. In 1977 Silverstein[160] updated the spectrum of PVSG defined PTH *vs* AMM. AMM or PMF is a clinico-pathological entity not preceded by any other PVSG defined MPD ET, PV, CML, or preleukemia (MDS)[8,160] and characterized by various degrees of anemia, splenomegaly, leukoerythroblastosis, with tear drop-shaped erythrocytes, and dry tap on BM aspiration due to various degrees of MF or osteomyelofibrosis. PMF or AMM patients are usually of age between 50 to 80 years, have enlarged spleens, a leukoerythroblastic blood reaction, striking teardrop poikilocytosis and dry tap on bone marrow aspiration. In the studies of Thiele and Spivak the mean age of advanced PMF or AMM is above 60 and around 70 years[161,162]. According to our experiences as documented in 1992[31] this clearly indicate that masked MPD preceding PMF must have been overlooked for 10 to 15 years as the consequence of extremely crude criteria for AMM (PMF) of anemia, splenomegaly and myelofibrosis. According to Silverstein *et al*[160] a typical AMM bone marrow is fibrotic in most cases, hypocellular in 85%, normocellular in 5% and hypercellular in 10%. Anemia due to ineffective erythropoiesis developed in about 60% of AMM patients within 5 to 10 years and thrombocytopenia and leuko-penia related to hypersplenism was seen in 30% and 14% of AMM patients[160]. The diagnostic criteria PVSG defined PMF or AMM have not been changed by the 2001 WHO using the term CIMF[75] and the 2008 WHO using the term PMF[76].

In the 2012 Tefferi *et al*[163] updated one thousand consecutive patients with PMF seen at Mayo Clinic between November 4, 1977, and September 1, 2011. The international prognostic scoring system (IPSS), dynamic IPSS (DIPSS), and DIPSS-plus were applied for retrospective risk stratification. Separate analyses were included for patients seen at time of referral (n = 1000), at initial diagnosis (n = 340), and within or after 1 year of diagnosis (n = 660). Anno 2012, 592 deaths and 68 leukemic transformations (6.8%) have been documented. Parameters at initial diagnosis *vs* time of referral included median age (66 years *vs* 65 years), male sex (61% *vs* 62%), red cell transfusion need (24% *vs* 38%), hemoglobin level less than 10 g/dL (38% *vs* 54%), platelet count less than 100× 10^9/L (18% *vs* 26%), leukocyte count more than 25 × 10^9/L (13% *vs* 16%), marked splenomegaly (21% *vs* 31%), constitutional symptoms (29% *vs* 34%), and abnormal karyotype (31% *vs* 41%). Retrospective screening for mutational frequencies anno 2012 were 61% for JAK2^{V617F}, 8% for MPLW515, and 4% for IDH1/2. DIPSS-plus risk distributions at time of referral were low in 10%, intermediate-1 in 15%, intermediate-2 in 37%, and high in 37%. The corresponding median survivals of DIPSS-plus low, intermediate 1 and 2 and high were 17.5, 7.8, 3.6, and 1.8 years *vs* 20.0, 14.3, 5.3, and 1.7 years for patients younger than 60 years of age. Compared with both DIPSS and IPSS, DIPSS-plus showed better discrimination among risk groups. Five-year leukemic transformation rates were 6% and 21% in low- and high-risk patients, respectively. Tefferi *et al*[163] concluded in 2012 that prognosis in MF should be assessed after a period of clinical observation after diagnosis rather than at diagnosis. It seemed that about 50% are to be treated conventionally, about 25% are candidates for allogenic stem cell transplant and roughly 20% can be treated with a JAK2 inhibitor (Ruxolitininb)[163].

JAK2/MPL-negative CALR mutated ET and MF: From Tefferi to Green and Kralovics

The molecular etiology of JAK2/MPL wild type ET and MF remained elusive untill two groups independently discovered the calreticulin (CALR) mutations in MPN patients with nonmutated JAK2[164-166]. Klampf *et al*[164] in Vienna Austria first described the occurrence of calreticulin (CALR) mutation in 78 of 311 (25%) ET patients and in 72 of 203 (35%) MF patients and in none of 382 PV patients. CALR mutations are mutually exclusive with both JAK2^{V617F} and MPL^{515} mutations: 195 (67%) of 289 JAK2 wild type ET and 105 (80%) of 120 195 carried one of the CALR mutations. In 150 patients with the CALR mutation for whom matched T-lymphocyte DNA was available, the CALR mutations were somatic. The CALR mutation was not found 45 CML, 73 MDS, 64 chronic myelomonocytic leukemia (CMML) and 24 RARS-T patients except that 3 SF3B1 positive RARS-T patients carried a CALR mutation. Klampf *et al*[164] detected a total of 36 types of somatic mutations (insertions and deletions) in exon 9 of the *CALR* gene encoding the C-terminal amino acids of CALR protein. Only 3 patients were homozygous. Among 1235 ET and MF patients 63.4%, 4.4% and

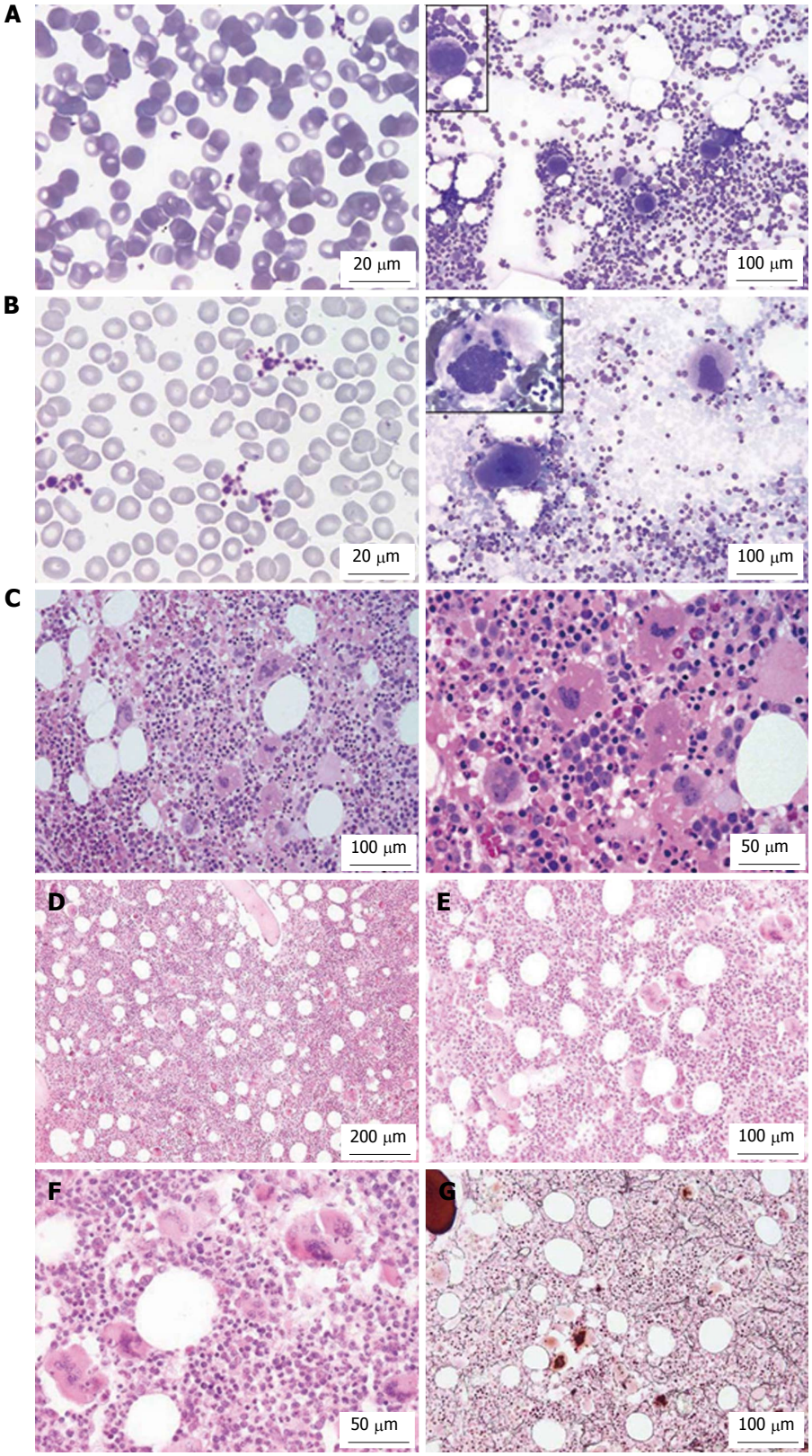


Figure 16 JAK2^{V617F} positive hypercellular essential thrombocythemia (platelets 453 × 10^9/L) in a case of portal vein thrombosis[94]. Large platelets in peripheral blood smear (B left) as compared to control (A left), bone marrow smear with large megakaryocytes with multilobulated nuclei (B right) as compared to control (A right) and hypercellular bone marrow due to increased erythropoisesis and granulopoiesis with pleomorphic megakaryocytes (C-E) similar as in typical polycythemia vera case 9 (F) with a hypercellular bone marrow picture due to increased erythropoiesis and granulopoiesis and slight increase of reticuline fibers grade 1 (G).

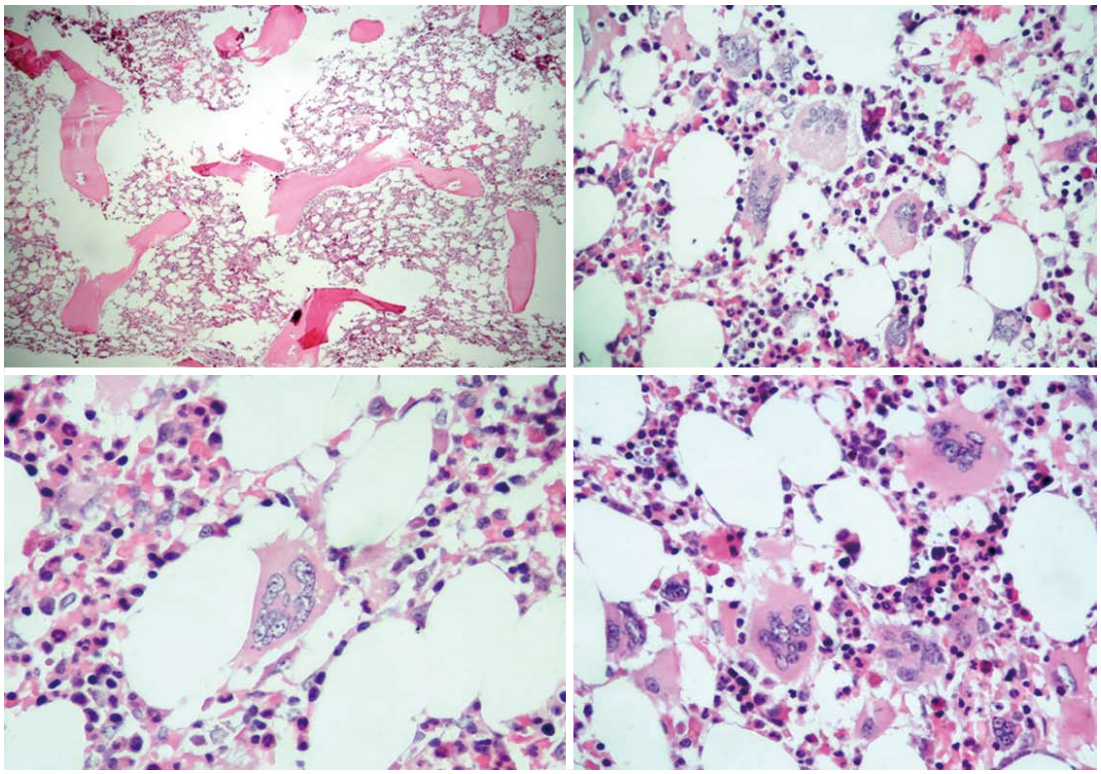


Figure 17 Bone marrow histology in normocellular essential thrombocythemia carrying the MPL^{W515L} mutation showing giant megakaryocytes with hyperlobulated stag-horn like nuclei charateristic for essential thrombocythemia the MPL^{W515L}/K mutation.

23.5% carried the JAK2^{V617F}, MPL^{515} and CALR mutation respectively, and in 8.8% none of these clonal markers (triple negative) was detected[164]. Life expectance was significantly longer in CALR mutated MF patients as compared to those with a JAK2^{V617F} or MPL^{515} mutation but the mean age at diagnosis of CALR mutated MPN patients was about 10 years younger than the JAK2V617F mutated MPN patients[164,165]. Evolution into MF during very long term follow up was equally high in CALR mutated ET as in JAK2 mutated PV (about 20% after 20 years follow up). CALR muated MPN patients had higher platelet counts, normal to low normal hemoglobin and white blood cells counts and a lower incidence of major thrombotic events simple because ist lacks PV features[164,165]. Nangalia *et al*[166] in the United Kingdom independently found somatic CALR mutations in 110 of 158 JAK2 and MPL wild type MPN, including 80 of 112 (70%) ET patients, 18 of 32 (56%) MF patients. CALR exon 9 mutations were found in 26 of 31 (84%) patients with JAK2/MPL wild type MF. CALR exon 9 mutations were absent in all 120 patients who had JAK2 or MPL mutations. CALR mutations were CALR mutations were identified in 10 of 120 (8%) MDS patients (RA in 5 of 53, RARS in 3 of 27 and RAEB-T in 2 of 27), and in one patient each with CMML and atypical CML. Patients with CALR mutations in the UK study had a significantly higher incidence of transformation of ET to MF than did those with JAK2 mutation (P = 0.03 Fisher's exact test)[166]. No CALR mutations were found in control samples, lymphoid cancers, solid tumors, or cell lines[111]. All CALR mutations identified in 148 patients were indels with 19 distinct variant: 14 deletions, 2 insertions and 3 complex indels, which generated a +1 base-pair frameshift, which result in a mutant protein with a novel C-terminal with the consequence that a large proportion, or almost all negatively charged amino acids and calcium binding sites are lost. More than 80% of the more than 30 identified indels involved either the type Ⅰ 52-bp deletion or the type Ⅱ 5-bp insertion. Patients with rare homozygous CALR mutation (all with the 5-bp insertion) were also identified[167]. The mechanism by which the CALR gain of function mutation selectively drives the neoproliferation of megakaryopiesis and granulopoiesis and not erythropiesis is unexplained and under investigation[167].

In the retrospective study from the Mayo Clinics Rochester United States of 254 evaluable WHO-defined MF patients the JAK2-, MPL- and CALR-mutations were detected in deep freeze sample in 58%, 8.3% and 25% respectively, and 8.7% were triple negative[168]. The retrospectively calculated median overall survival durations of 83 CALR-, 21 MPL-, and 147 JAK2-mutated MF cases and in 22 triple negative MF cases were 8.2, 4.1, 4.3 and 2.5 years respectively. As compared to CALR wild type MF, CALR-mutated MF patients were younger, had higher platelet count, lower leukocyte count, were less anemic with lower DIPSS-plus score. CALR-mutated MF patients had a favorable impact on median survival as compared to CALR-negative MF patients whether the addional sex combs like 1 (ASXL1) loss-of function mutation is negative or positive. The

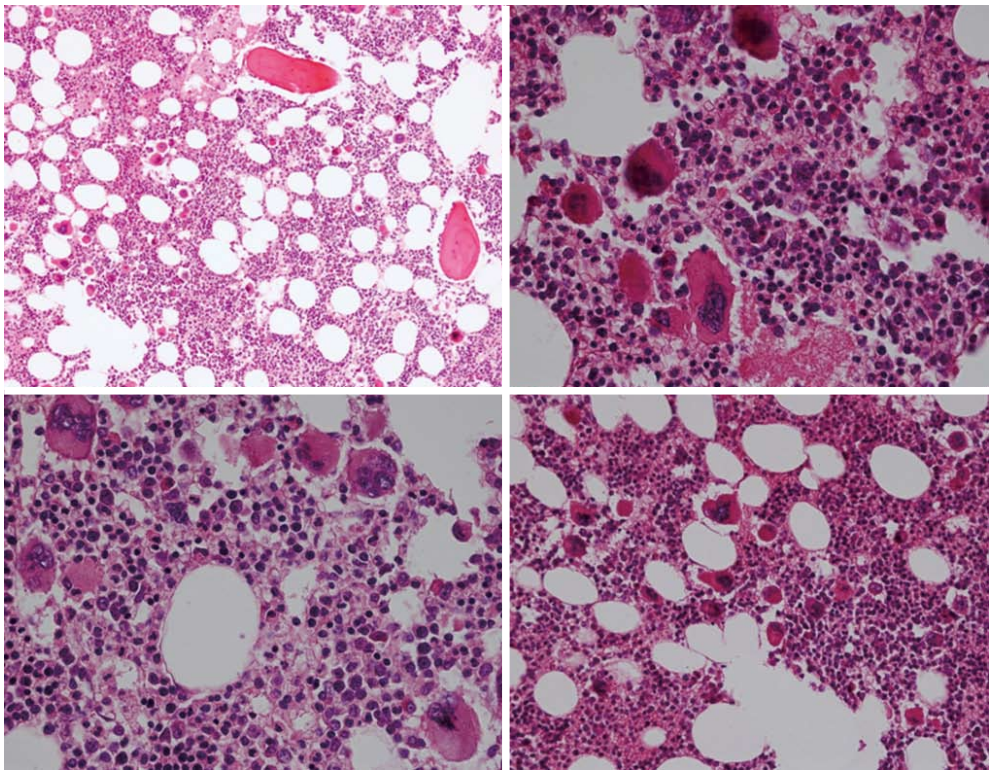


Figure 18 Clinical case of JAK2/MPL negative and calcireticulin positie essential thrombocythemia who present with aspirin responsive platelet thrombophilia, normal values for hemoglobin. Hematocrit and erythrocytes, platelet count of 1832 × 10^9/L and slight splenomegaly (16 cm lenght diameter on echogram). Bone marrow histology is hypercellular with relative decrease of erythropoisis, dense cluster of immature megakaryocytes with hypolobulated nuclei consistent, and no increase of reticulin fibrosis consistent with a typical primary megakaryocytic granulocytic myeloproliferation (Table 10) bone marrow.

median overall survival was 2.3 years in 55 CALR-negative/ASLX1-positive as compared to 5.6 years in 126 CALR-negative/ASXL1-negative MF patients. The median survival was 7 years in 20 CALR-positive/ASXL1-positive MF patients as compared to 9.6 years in 126 CALR-positive/ASXL1-negative MF patients[168].

PMGM bone marrow histology in JAK2 wild type CALR mutated ET and MF

The bone marrow histology in 6 consecutive newly diagnosed CALR mutated ET and early MF revealed a typical PMGM picture showing dysmorphic megakaryocytes with definite abnormalities of maturation with bulky (bulbous) hyperchromatic nuclei and some disturbances of the nuclear cytoplasmic ratio (Table 11)[77,78], which are not seen in JAK2 wild type MPL^{515} mutated ET also not in prefibrotic JAK2^{V627F} mutated ET, ET/PV, EMGM[77,78]. Bone marrow findings in JAK2/MPL wild type PMGM are consistent with hypercellular ET with pronounced thrombocythemia as the presenting feature of CMGM described by Georgii *et al*[35,36] (Table 4). These features CMGM or PMGM are similar to those reported by Thiele as prefibrotic PMF in the 2008 WHO classification[97-100]. Representative bone marrow histology findings of typical cases of CALR positive ET (Figure 18) and MF (Figure 19) show dense cluster of immature megakaryocytes with the typical picture of CIMF according to 2001 WHO[75] and PMF according to 2008 WHO classification[76]. The finding of CALR mutation as the driver mutation has been confirmed in Belgium in 40 of 64 JAK2 wild type MPN (ET or MF) and 24 MPN cases were JAK2/MPL/CALR triple negative. The clinical presentation, laboratory and molecular findings and the bone marrow histology features are under investagation and will be compared with 50 cases of JAK2^{V617F} mutated ET, PV and EMGM.

2015 WHO-CMP criteria for classification and staging of MPN

The 1986 PVSG defined ET overlooks the ECMP and WHO-CMP defined ET with platelet count between 400 to 600 × 10^9/L, which comprises 30% of very early stage PV in various MPNs[169]. In 2008 the WHO reduced the minimum platelet count required for the diagnosis of ET to 450 × 10^9/L[76]. The 2015 WHO-CMP criteria clearly define JAK2^{V617F} positive normocellular ET prodromal PV, prefibrotic classical PV, early fibrotic PV, PV complicated by myelofibrosis, significant myeloid metaplasia of the spleen (splenomegaly and related constitutional symptoms) (Table 11, Figure 20). Within the JAK2^{V617F} MPN entities, the JAK2^{V617F} positive hypercellular ET is associated with clustered pleomorphic megakaryopoiesis (not cloud-like), increased granulopoiesis and relative decrease of erythropoiesis (EMGM, masked PV). The EMGM entity or masked PV is situated clearly between the normocellular ET and post-ET myelofibrosis carrying the JAK2^{V617F} mutation (Figure 20)[77,78]. Campbell *et al*[170] assessed the clinical features in the cohort of 806

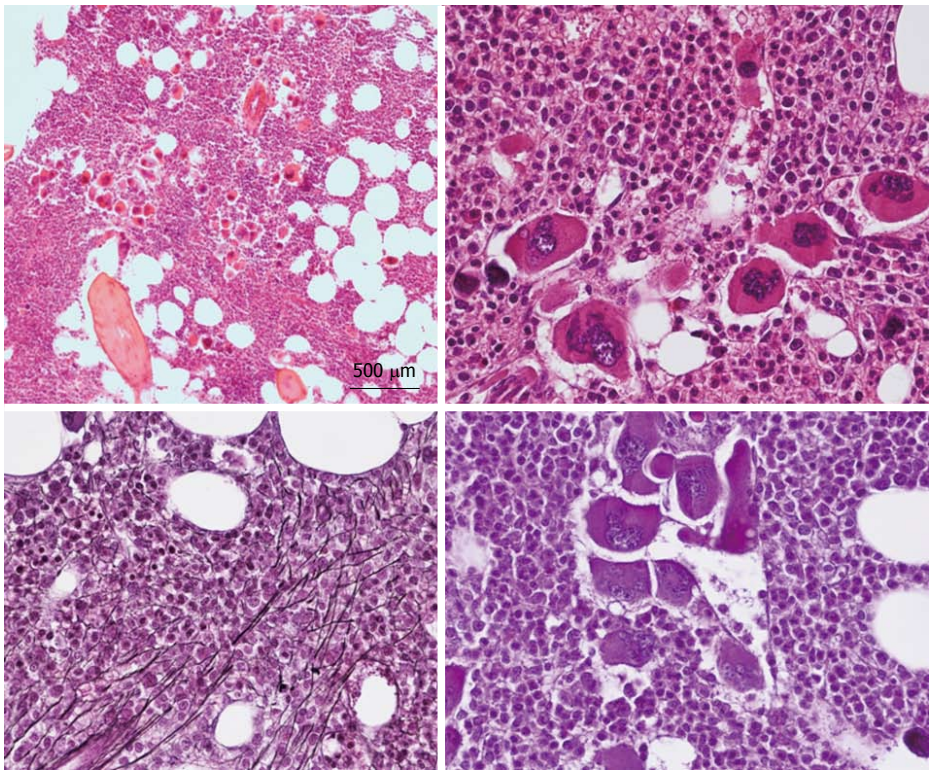


Figure 19 Clinical case of calcireticulin positive myelofibrosis: Hemoglobin 11.2 g/dL, hematocrit 0.33, leukocytes 9.2 × 10^9/L, platelets 347 × 10^9/L, lactodehydrogenase 1393 U/L, and the presence of tear drop erythrocytes, poikolocytosis and polychromasie in a peripheral bloodsmear, and hypercellular bone marrow with relative decrease of erythropoisis, dense cluster of immature megakaryocytes with hypolobulated nuclei consistent, and reticulin fibrosis grade 2 consistent with bone marrow histology features similar to World Health Organization-defined primary myelofibrosis, but distinct from JAK2^{V617F} mutated essential thrombocythemia and polycythemia vera, and distinct from MPL^{515} mutated essential thrombocythemia (Figures 17).

PVSG defined ET patients subdivided in 414 JAK2^{V617F} positive and 362 JAK2 wild type ET and evaluated the bone marrow features in 393 ET patients[171]. JAK2^{V617F} positive ET patients had multiple features of PV with significantly higher hemoglobin, lower serum EPO and ferritin, higher neutrophils, bone marrow erythrocytosis and granulocytosis, more venous thrombosis and a higher rate of polycythemic transformation. PVSG defined JAK2 wild type ET had significant higher platelet counts (962, range 668-1535 × 10^9/L) than JAK2^{V617F}-positive ET (846, range 632-1222 × 10^9/L). In the PT-1 study, bone marrow trephine of 209 JAK2^{V617F} positive and 184 JAK2 wild type ET was independently assesed by 3 hematopathologists who did not know the JAK2 mutation status[171]. The overall cellularity was significantly increased in JAK2^{V617F} mutated ET, indicating at least in part of them an increased erythroid and/or granulocytic cellularity, which are features of PV or EMGM (masked PV).

Pich *et al*[172] prospectively analyzed histological changes in diagnostic bone marrow biopsy from 2006-2010 of 103 newly diagnosed 2008 WHO defined ET patients. Bone marrow features in 44 JAK2 wild ET cases revealed prominent clusters of large megakaryocytes with stag-horn nuclei, less micromegakaryocytes and much less erythroid hyperplasia similar to normocellular "true" ET and hypercellular "false" ET (Figure 17). In contrast, 59 JAK2^{V617F} positive ET patients (Figure 16) revealed a PV phenotype with higher hemoglobin, hematocrit, erythrocytes and bone marrow features with increased cellularity frequently some hyperplasia of erythroid and myeloid lineages and pleomorphic megakaryocytes very similar as in WHO-ECMP defined ET and PV. The mean and median JAK2^{V617F} mutation burden in WHO-ECMP defined ET was 14.4% and 8.7% respectively. Interestingly LDH (604 + 132) and spleen size (15.4 + 4.9) in 16 cases with a JAK2^{V617F} mutation load above 12.5% were significantly increased as compared to normal LDH (386 + 94) and normal speen size (11.2 + 2.1) in 37 cases with a JAK2^{V617F} mutation load below 12.5%[172].

The 2015 WHO-CMP classification and staging of patients with MPN will be very helpful in predicting and documenting prospectively the natural history of JAK2^{V617F} mutated ET, PV and EMGM patients (Table 11), *vs* MPL^{515} mutated ET, *vs* JAK2 wild type CALR mutated ET and MF associated with PMGM (Figure 20). The primary involvement of basic researchers, laboratory scientists, molecular biologists and clinicians as well as pathologists are essential to document the natural history at the clinical molecular and bone marrow level to demonstrate that scrutinized and integrated clinical, laboratory, molecular and pathological approaches and intense communications amongst clinicians, molecular biologists and pathologists are warranted in prospective diagnostic and managements studies. The 2015 WHO-

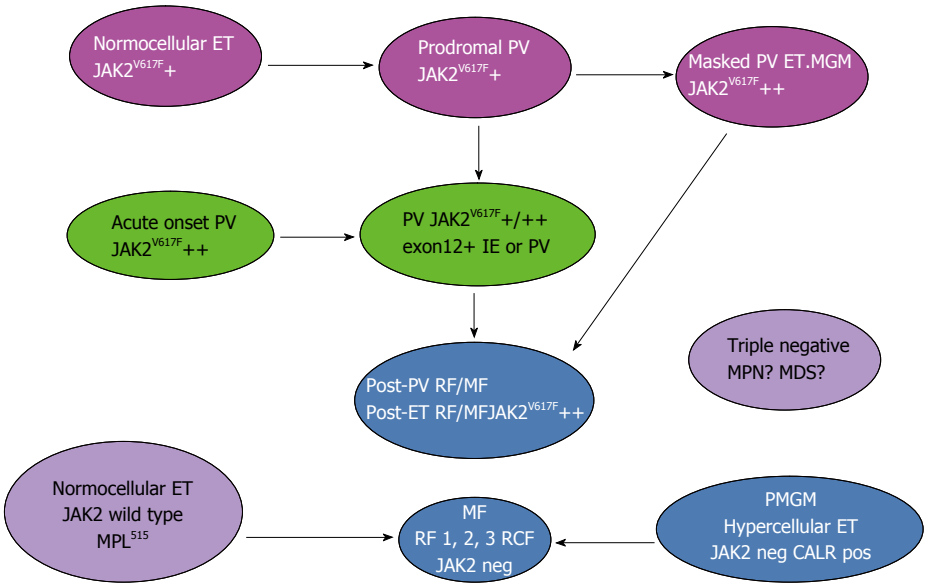


Figure 20 World Health Organization-clinical molecular and pathological myeloproliferative neoplasms classification and transitional states. ET: Essential thrombocythemia; PV: Polycythemia vera; MGM: Megakaryocytic granulocytic myeloproliferation; IE: Idiopathic erythrocythemia; RF: Reticulin fibrosis; MF: Myelofibrosis; RCF: Reticulin collagen fibrosis; CALR: Calreticulin; PMGM: Primary megakaryocytic granulocytic myeloproliferation.

CMP criteria surely will have important implications in choosing proper targeted treatment options for the management and prevention of thrombotic and bleeding complications and serious complications of progressive MPN disease burden in prodromal PV and overt PV (Table 11). Proper staging of PV in terms of JAK2^{V617F} mutation load, and MPN disease burden including splenomegaly, constitutional symptoms including itching, bone marrow histology and grading of myelofibrosis is of huge importance since it has significant implications for a non-leukemogenic or the least potential leukemogenic treatment options in low, intermediate and high risk PV patients (Table 11)[77,78]. A primary rigid venesection regimen aiming at a hematocrit around and below 0.40 seems to us better than the target of < 0.45 in males and < 0.42 in females on top of low dose aspirin for the control of activated platelets in MPN. According to our extended experiences, this strategy in stage zero, 1 and 2 PV patients (Table 11) will reduce the cumulative incidence of minor and major thrombosis from above 50% to less than 2% per patient/year during long-term follow-up.

The rational for using IFN-α as the first-line treatment option in newly diagnosed PV-patients include its effectiveness to abate constitutional symptoms and to induce a complete remission, thereby avoiding phlebotomy, iron deficiency and macrocytosis associated with hydroxyurea[173-176]. Clinicians will be reluctant to postpone the use of hydroxyurea as long as possible or even life long in early stage PV[93,116]. Two studies show IFN-induced complete hematological responses within one year, and major molecular responses (MMR) were reached after a follow-up of 2 to 3 years in PV and ET patients[173,174]. The cumulative incidence of MMR was 14% at 2 years and 30% at 4 years follow-up in the study of Kiladjian *et al*[175]. Peglyated IFN-α-2a reduced the median JAK2-allele burden from 45% to 5% in 37 PV patients in the study of Kiladjian *et al*[175] and from 64% to 12% in79 PV and ET patients in the study of Quintás-Cardama *et al*[176]. Larsen *et al*[177] demonstrated that a complete molecular response may be reached, which was associated with normalization of bone marrow histology. MPN patients and their physicians should be cautious and attentive not to become too enthusiastic since the use of pegylated IFN-α-2a or -2b may be associated with significant side effects in about one third of PV patients[93,116]. We do know that a significant proportion of around 50% of early and intermediate stage PV patients are responsive to IFN with no minor or bearable side effects[116]. The misconception in the past was to start with too high dosages of IFN[93,173]. According to current insights, low dose pegylated IFN is the treatment of choice in intermediate stage PV patients and high risk PV in terms of high JAK2^{V617F} allele burden. Patients with progressive MPN disease, splenomegaly and constitutional symptoms are candidates for myelosuppressive (hydroxyurea) or myeloreductive (JAK2 inhibtors) treatment (Table 11)[173-180]. MF transformation of thrombocythemia in MPN of various molecular etiology has to be distinguished from the expansion of one dominant homozygous subclone, the selective advantage of which is likely to reflect additional cytogenetic, genetic or epigenetic lesions (Table 12). Such additional, acquired background biological factors on top of the JAK2, MPL and CALR driver causes of MPN will become of huge importance for the understanding of differences in prognosis and outcome.

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REVIEW

Storage lesion: History and perspectives

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Abstract

Red blood cell concentrates (RBCCs) are the major labile blood component transfused worldwide to rescue severe anemia symptoms. RBCCs are frequently stored in additive solutions at 4 ℃ for up to 42 d, which induces cellular lesion and alters red blood cell metabolism, protein content, and rheological properties. There exists a hot debate surrounding the impact of storage lesion, with some uncertainty regarding how RBCC age may impact transfusion-related adverse clinical outcomes. Several studies show a tendency for poorer outcomes to occur in patients receiving older blood products; however, no clear significant association has yet been demonstrated. Some age-related RBCC alterations prove reversible, while other changes are irreversible following protein oxidation. It is likely that any irreversible damage affects the blood component quality and thus the transfusion efficiency. The present paper aims to promote a better understanding of the occurrence of red blood cell storage lesion, with particular focus on biochemical changes and microvesiculation, through a discussion of the historical advancement of blood transfusion processes.

Key words: Ageing; Blood cells; Exosomes; Microparticles; Microvesicles; Proteomics; Storage; Transfusion

Core tip: This review paper puts in perspective the red blood cell storage lesion, which is a hot topic in transfusion medicine. Many different physiological and biochemical pathways are affected by cold storage, and stored red blood cells are clearly very different when compared to freshly drown erythrocytes. However, most of clinicians are lost in translation because experimental data and clinical data are divergent. Therefore, both fundamental, translational and clinical studies are needed in the near future to provide better care to our patients.

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INTRODUCTION

One major paradox of modern transfusion medicine is related to the fact that donated blood is not transfused directly from donors to patients, but rather undergoes processing for at least 1-2 d. Donated whole blood is most often fractionated into several different components: Red blood cells (RBCs) are packaged as red blood cell concentrates (RBCC) and stored at 4 ℃, the platelet component (PC) is stored at room temperature (22 ℃) under constant agitation, and therapeutic plasma is generally stored frozen until its use as fresh frozen plasma (FFP). Interestingly, in some emergency rooms, physicians ask for "resuscitation packs" that comprise four RBCCs, four FFP units, and 4-6 PC units. These components are then combined with the aim of recovering whole blood properties.

Decades ago, freshly drawn whole blood was transfused from donor to recipient. The earliest procedures involved blood infusion directly from the donor arm to the recipient arm. Following the development of anticoagulation and short-term preservation techniques, blood could instead be bottled and infused with no necessary contact between the donor and the recipient. Obviously, the reconstituted whole blood presently used in emergency wards is a completely different essential medical product. The modern process offers substantial improvements in terms of quality and immunohematology, and thus of safety. However, it also introduces some caveats that can be underestimated or mistakenly ignored in resuscitation regimens-particularly the possibility of storage lesion.

Here we discuss the various steps that have guided the acquisition of quality and safety over time, along with the supposed minimization of lesion. We start with a retrospective (historical) overview, followed by the natural history of storage lesion. Overall, this look-back aims to provide a better understanding of the clinical impacts of storage lesion in order to prevent unnecessary hazards.

A LOOK BACK AT BLOOD TRANSFUSION PRACTICES

The story of blood transfusion really begins in 1628 with the first description of the human blood flow system by the English physician William Harvey in the "*Exercitatio Anatomica de Motu Cordis et Sanguinis in Animalibus*". Based on this seminal advancement of human physiology knowledge, blood became viewed as the "river of life". Several physicians theorized that transference of blood from a healthy person to a sick person could produce curative effects, with targeted sicknesses spanning the spectrum from febrile state to mental disorders.

The injection device first used to attempt blood transfusion was developed in 1656 by the English architect and scientist Christopher Wren, who designed it to test the feasibility of conveying liquid poison into a dog bloodstream. The first blood transfusions are attributed to the British physician Richard Lower, who reportedly performed animal-to-animal and animal-to-human blood transfusions as early as in 1665. The French physician Jean-Baptiste Denis was the first to communicate his experience with animal-to-human blood transfusion, claiming in 1667 to have successfully used a transfusion of sheep blood to cure a 16-year-old boy suffering from fever. However, further attempts were unsuccessful, and one transfusion led to Denis being charged with murder-although this death was ultimately found to have been caused by the victim's spouse and not influenced by the transfusion itself. The controversy led to the 1675 edict of the Châtelet prohibiting blood transfusion in humans and restricting the practice to animal experiments in France.

Approximately one and a half centuries later, in 1829, the English obstetrician James Blundell established the conditions for successful human-to-human blood transfusions. He further documented several successful transfusions and contributed to the development of instruments for blood transfusion. Creite and Landois described the almost systematic lethal agglutination phenomenon observed with animal-to-human transfusion, leading the community to progressively abandon this practice. In the early 1900s, Karl Landsteiner investigated the interactions between blood serum and red corpuscles from different human individuals, leading to his discovery of three groups of blood.

Early blood transfusion was a surgical act involving the dissection of veins or arteries in both the donor and the recipient. End-to-end anastomosis of blood vessels was commonly performed using the Carrel's suture and Crile's cannula methods. Transfusions were thus performed arm-to-arm, meaning that donors had to be present next to the patients. The first direct blood transfusion (arm-to-arm) of World War I (WWI) was performed by the French physician Emile Jeanbrau in 1914. Despite the recommendations made by Hektoen in 1907, the donor and recipient bloods were not cross-matched to ensure their group compatibility, but the transfusion was luckily successful. And so were performed most transfusions during the war. Indeed, the watchword was to accept the risk of blood incompatibility issues rather than let people die from massive hemorrhage.

WWI created a need to dissociate the blood donor from the recipient. Technical development of transfusion devices allowed retrieval of blood from a donor and its injection in the recipient, but such procedures were

often unsuccessful due to blood clotting. In the early 1890s, citrate had been shown to interfere with the clotting process through calcium binding, and Wright[1] even declared that citrated blood appeared more suitable for transfusion. However, at that time, blood clotting was a minor issue compared with the not yet understood group incompatibility-related agglutination. Moreover, sodium citrate was considered highly toxic and thus unsuitable for human transfusion. In 1914, the Belgian physician Albert Hustin reconsidered the use of sodium citrate to prevent blood coagulation. In 1914-1915, Hustin in Brussels, Agote in Buenos-Aires, and Lewisohn in New York each independently performed successful transfusions of citrated blood to human. This discovery allowed the physical dissociation of recipient from the donor, but it could not be used on a large scale until the end of the war.

Sodium citrate kept blood liquid for up to a few days but not for longer periods, and long-term blood storage was not yet feasible. In 1916, Rous and Turner[2,3] achieved the first success in erythrocyte conservation, reporting reasonably low hemolysis in rabbit RBCs that were stored in an icebox for four weeks in a solution composed of sodium citrate and glucose. However, such stored RBCs could only be efficiently transfused back to rabbit when the storage duration was 2 wk or less[3], highlighting the problem of storage-related RBC aging. Thanks to this method, in 1918, Oswald Robertson[4] reported the efficient storage, transport, and transfusion of 22 "units" of human RBCs stored for 26 d during rush periods at casualty clearing stations. The set-up described by Robertson[5] can be considered the world's first blood bank. He also developed a specific bottle equipped with a negative or positive pressure pump device for blood collection in citrate solution or infusion of the citrated blood to the recipient[5].

Due to its duration and the number of involved countries, WWI became a theater of rapid developments and experimentations in blood transfusion. From isolated attempts to rejuvenate life, blood transfusion became part of the routine medical care for soldiers suffering from hemorrhage. After WWI, transfusion-related technical development was limited. Direct and indirect transfusion co-existed. In arm-to-arm transfusion, the control of transfused blood volumes was improved, *e.g*., by the apparatus of Tzanck (1925) or the pump of DeBakey (1935). The 1930s saw continued use of existing devices for indirect transfusion, *e.g*., syringes, paraffin-coated tubes, and temporary storage bottles-with the primary improvement being more effective use of sodium citrate. Post-WWI transfusion-related developments mainly focused on the generation of methods applicable in a large-scale practice, and the identification and organization of potential donors for peace times.

The period between WWI and WWII was especially marked by the fact that this emergency procedure entered the field of medicine with extended indications in trauma, surgery, and obstetrics. As blood donation had become routine practice with soldiers, it began to further penetrate civilian life. The growing extent of blood transfusion practice required more donations and greater organization. Thus, the 1920s witnessed the creation of the first blood transfusion centers, followed by the establishment of blood banks in the late 1930s. In 1936, Spain established the world's first blood bank during its civil war. This was rapidly followed by the appearance of blood banks in the United States of America and in other European countries shortly before WWII.

In 1939, Levine *et al*[6] reported the case of a group O patient whose serum induced iso-agglutination (intragroup erythrocyte agglutination) in 80% of tested group O blood samples. One year later, Karl Landsteiner and Alexander Wiener[7] highlighted the existence of the Rhesus factor, showing that the serum of rabbits immunized with the blood of Rhesus monkeys (Macaca mulatta) was reactive to nearly 85% of blood samples from humans (Caucasian), in a manner independent of ABO blood group. Landsteiner *et al*[7] further confirmed that this antigen caused the majority of ABO-compatible transfusion reactions. This discovery greatly improved the safety of the blood transfusion process for recipients-apart from the possibility of transmissible infections.

In 1940, the process of fractionating plasma into different protein constituents gained attention. In particular, Edwin Cohn[8,9] established the basis for plasma fractionation with two papers introducing the separation of equine or bovine serum upon equilibration across membranes using ammonium sulfate or ethanol dilutions with controlled pH, ionic strength, and temperature. The second paper also described a detailed procedure to obtain 50 g of albumin from 2 L of plasma[9]. Cohn's method allowed the successful production and use of therapeutic lyophilized albumin units during WWII.

Blood preservation saw another substantial improvement in 1943. The alkaline pH of glucose-containing anticoagulant preservative solution led to caramel production during autoclaving, thus requiring separate autoclave heating procedures for the trisodium citrate and glucose solutions, followed by aseptic mixing. To simplify this process, Loutit *et al*[10] tried to counterbalance sodium citrate with citric acid. Testing preservative solutions with different sodium citrate/citric acid ratios, they monitored caramel formation and parallel glucose loss, as well as screened blood storage for four weeks at 3 ℃-7 ℃ and examined end-storage hemolysis. Satisfactory solutions were further analyzed for post-transfusion *in vivo* survival and several biochemical parameters-such as spontaneous hemolysis, osmotic fragility, pH, glucose, potassium, and formation of methemoglobin-with comparison to the Rous-Turner and the English Medical Research Council (MRC) preservative solutions in use at the time. They concluded that acidified citrate-glucose (ACD) preservative solutions were satisfactory for blood storage and were recommended for the following

reasons: (1) better survival in recipient circulation; (2) low caramel formation in the autoclaved whole solution; (3) no adverse outcomes during and following transfusion of ACD-stored blood; and (4) an absence of substantial storage-related methemoglobin formation compared with the currently used preservative solutions. ACD blood preservative solutions allowed blood storage for three weeks (based on 24-h post-transfusion survival) and remained in use until the 1957 introduction of phosphate by Gibson[11], which allowed blood storage for up to four weeks with increased levels of 2,3-DPG[12].

The 1950s witnessed a critical storage-related technical development. In 1952, Walter *et al*[13] presented the utilization of a closed system of plastic bags for blood collection, preservation in ACD, and transfusion. Compared to glass bottles, the plastic bag system had the obvious advantages of reduced bacterial contamination (no contact with air), lighter weight, shock resistance, and ease of storage in refrigerators. In a 1954 publication from the army medical service, Artz *et al*[14] reported the use of plastic bags for blood transfusion during the war of Korea. It was determined that blood stored in such bags had lower plasma potassium levels than blood stored in glass bottles. In fact, recent research shows that the plasticizer diethylhexyl phthalate is released from blood bags, and plays a beneficial role in limiting microvesiculation of RBC membranes[15]. However, in the 1950s, plastic bags were not welcome on battlefields. Gravity-related blood infusion to the recipient was considered inconvenient because "about 30 percent of the blood given in a forward surgical hospital must be given under pressure", and although plastic bags could be squeezed to force blood out, "it was impractical to assign one corpsman to each bag when 8 or 10 pressure transfusions were being given simultaneously to various patients"[14].

The next improvements of blood storage involved the introduction of adenine as a constituent of preservative solutions, as proposed in 1962 by Nakao *et al*[16]. This research group demonstrated that adenine and inosine enabled ATP regeneration and shape modifications of long-stored RBCs[17-19], and that ATP level directly impacts RBC *in vivo* viability[20]. Citrate phosphate dextrose adenine solutions (CPDA-1 and CPDA-2) were licensed for use in the United States during the late 70s/early 80s, although transfusion medicine had already shifted to the use of packed RBC concentrates. The first additive solution used for storage of packed RBC units was sodium-adenine-glucose (SAG)[21], which was further modified by addition of mannitol (becoming SAGM) to reduce end-storage hemolysis[22]. Other additive solutions were derived from the original SAG and are currently used worldwide, including anticoagulant solution (AS)-1, AS-3, and AS-5 in the United States and Canada, and MAP in Japan. The guidelines of 75% recipient survival at 24 h post-transfusion and less than 1% (sometime 0.8%) hemolysis allow RBCC storage in SAGM or derivatives for up to six weeks at 4 ℃.

The most recent improvement in blood transfusion involves the removal of leukocytes before or during whole blood processing. Leukoreduction is performed by removal of the buffy coat layer after whole blood centrifugation and/or leukofiltration. This is done because cold-induced leukocyte lysis is believed to damage the stored RBCs. Moreover, leukocyte depletion prevents virus transmission from the donor to the recipient, and inflammatory-like side-effects such as febrile non-hemolytic transfusion reactions and human leukocyte antigens (HLA) immunization[23]. The ideal duration of RBCC unit storage is debatable[24].

CURRENT COLLECTION AND STORAGE OF RBC CONCENTRATES

Many efforts have contributed to improving blood transfusion processes, including advancements in procedures for collection, storage, and infusion. Nowadays, blood components are obtained either by processing whole blood donations or by collecting individual components *via* apheresis-driven donation of the required fraction(s). Managing each blood component type separately allows optimized storage of each component in accordance with its intrinsic properties. Moreover, the various labile blood components can be transfused independently of each other, as each can be used to treat different pathologies. Re-transfusion of RBCs can correct anemia secondary to hemorrhage, medullar insufficiency, and anomalies of hemoglobin or erythrocyte membrane synthesis. PCs are transfused in cases of hemorrhagic disorders, and plasma units are used to treat complex hemostatic disorders.

RBCC TRANSFUSION EFFICACY AND CLINICAL OUTCOMES

RBCC efficiency is quite difficult to evaluate because this therapy was introduced before evidence-based medicine was formally established. Transfusion is almost unanimously considered a lifesaving procedure, with many advertisements promoting blood donation stating that "Blood saves lives". However, this cannot be taken at face value. It must also be considered that, in rare cases, transfusion may harm or even kill the recipient. Moreover, the increasing availability of alternative treatments mean that transfusion is no longer a simple matter of "life or death", but more often a situation in which transfusion provides a more effective or rapid treatment. Specialists in transfusion medicine tend to argue that transfusion is beneficial, while other physicians cast more doubts on the procedure and express concerns regarding on transfusion-linked hazards. It is important to achieve a realistic view of the benefit-hazard ratio of transfusion processes.

Blood transfusion can be associated with adverse events occurring during the process (transfusion

reaction) or during the 24-48 h following transfusion (post-transfusion hazards). The severity of such events can range from mild to severe and even life threatening. Different transfusion-related adverse events can be classified based on the causative pathogen (immune or inflammatory effector or infectious microbe), the type of reaction (hemolytic or not), and the time from transfusion to event occurrence (immediate *vs* delayed). Reported serious adverse events include hemolytic transfusion reaction, incorrect blood component transfused, post-transfusion purpura, transfusion-associated circulatory overload, transfusion-associated dyspnea, transfusion-related acute lung injury, transfusion-associated graft-versus-host disease, and transfusion-transmitted infection. Various implemented hemovigilance systems show changed in the incidences of various serious adverse events over time, including an almost complete disappearance of post-transfusion purpura and an important decrease of the reported cases of transfusion-related acute lung injury (with the exclusion of transfusions of plasma from women). Transfusion-related events can also include an increased length of hospitalization, induction of defective post-operative ventilation, deep venous thrombosis, troublesome respiratory syndrome, multiple organ dysfunction syndrome, or even death.

Several studies seem to find that transfusion was more strongly associated with complication risks than with benefits. However, these investigations did not account for several highly important factors, including patient age, severity of the illness requiring blood transfusion, therapeutic regimen, blood group, and the number of blood units being transfused. Thus, the suggestion that blood transfusion carries a great risk may be biased by adverse outcomes that are actually due to a bad health context. Similarly, a greater need for transfusion is associated with a more critical situation and higher morbidity risk. However, other studies have reported no effect of RBCCs storage duration[25-32], and a few have even found worse adverse outcomes associated with transfusion of fresher blood[33]. In their 2008 meta-analysis, Marik *et al*[34] reviewed the relationship between transfusion and the occurrence of adverse events among critically ill patients. Data from 45 studies (approximately half retrospective and half prospective cohorts) emphasized a link between RBCC transfusion and increased morbidity and mortality in 93% of the cases.

The potential role of blood transfusion in adverse outcomes has also been investigated with regards to the age of the transfused blood products, *i.e.,* the duration of storage before use. In 1993, blood storage duration was first questioned in relation to the biochemical efficacy of transfusion, *e.g.*, oxygen delivery[35]. In 1997, Purdy *et al*[36] performed a retrospective study of septic patients (admission diagnosis) that included the first reported association between blood storage duration and survival. They found that survival did not significantly differ in relation to age, sex, absolute number of transfused RBCCs, or length of the patient's stay at the ICU prior to receiving a transfusion; however, the mean age of the RBCCs transfused to survivors was significantly lower than for non-survivors (17 d *vs* 25 d of storage in CPDA-1, respectively). Notably, the survivors received a high proportion (85%) of RBCCs stored for less than 10 d, while non-survivors received a high proportion (76%) of RBCCs stored for more than 20 d. Blood groups were not taken into account. In another retrospective study, Vamvakas *et al*[37] highlighted an association between wound infection or pneumonia and the storage duration of transfused RBCs in patients undergoing coronary artery bypass graft surgery. Following the introduction of leukoreduction, several authors reported that the process had beneficial effects on known adverse outcomes[38,39]. Most results impute complications to long-stored RBCs[40-45]. In 2008, Koch *et al*[46] reported that aged RBCCs (more than 14 d of storage) were negatively associated with poor outcomes among pediatric cardiac surgery patients-a finding that had a particularly high impact on the transfusion medicine community. To date, many studies in different countries have examined the use of blood products-with various processing methods and in different pathological cases-to investigate whether long-stored blood components impact transfusion-related adverse clinical outcomes.

The reliability of these results varies based on how the studies were conducted, for example, the number of studied cases; control conditions; data correction with respect to confounding patient-dependent parameters (*e.g.,* age, sex, blood group, illness severity, number of transfused blood units); and study design (*e.g.,* retrospective *vs* prospective, randomized or not, blinded or not, and dichotomization approach or not). It has been reported that conclusions depend on the number of transfused units[47]. It appears that many post-transfusion complications were due to immunomodulatory effects attributed to residual leukocytes in RBCCs[48,49]. Moreover, leukocyte depletion has been recently shown to improve RBC storage in terms of biochemical alterations[50].

The available literature comprises very differently conducted studies, making it difficult to perform meaningful meta-analyses. One particular issue concerns geography - for example, the processes for blood product preparation differ between America and Western European countries. Van de Watering[33] has proposed that meta-analyses could be facilitated by more complete reporting of the characteristics of production and storage of RBCs, such as donation type, overnight incubation at room temperature, leukocyte reduction method, preparation of RBC component, and types of bags and additive solution used. Overall, the present data suggest that the duration of blood storage is probably less stringent than initially thought except in specific cases: Namely, extracorporeal circulation and (with less certainty, because there is no suitable comparator) in infants. The European Guide currently recommends giving fresh blood to fetuses, neonates,

Table 1 Overview of the storage lesions

	Lesions	Reversible or irreversible[1]
Biochemical	Loss of metabolic modulation	Reversible
	Accumulation of lactate and pH drop	Reversible
	Ion leakage (K^+)	Irreversible
	Decrease of antioxidant defenses	Reversible
	ATP-dependent protein function	Reversible
	Protein oxidation (sulfenic acid)	Reversible
	Protein oxidation (carbonylation)/degradation	Irreversible
	Membrame proteins (band 3 dimerization/accumulation of oxidized proteins)	Irreversible
	Hemolysis	Irreversible
	Lipid oxidation	Irreversible
Morphological	Exposure of senescence markers (phosphatidylserine)	Irreversible
	Shape change	Reversible or irreversible
	Reduced deformability	Irreversible
	Microvesiculation	Irreversible

[1]Either *in vivo* or *in vitro*.

and infants, despite the lack of evidence-based studies. This remaining uncertainty urges the transfusion community to address the precise issue of the age of blood. The ongoing ABLE trial will provide some but not all of the needed information.

RBC STORAGE LESION

While the clinical relevance of aged RBCC transfusion on recipient adverse outcomes remains uncertain, there is no doubt that changes at the cellular and molecular levels increase with the storage duration[51,52] (Table 1). During RBCC storage at cold temperatures (4 ℃ ± 2 ℃), three types of lesions occur at the cellular and intracellular levels: Biochemical lesion, oxidative lesion, and biomechanical lesion.

Erythrocytes undergo biochemical lesion, altering their metabolism. Anaerobic glycolysis occurs in the cytoplasm of RBCs due to the absence of mitochondria. Consequently, energy metabolism through glucose consumption leads to lactate production, which accumulates in the RBC storage solution[53] and induces its acidification[54]. This metabolism-related decrease of pH inhibits the glycolysis rate, and affects the 2,3-diphosphoglycerate (2,3-DPG) level through the activities of diphosphoglycerate mutase and phosphatase. After two weeks of storage, RBCs reportedly show 2,3-DPG depletion[55]. Low 2,3-DPG levels increase the affinity of hemoglobin for oxygen, inhibiting its release and thus altering the oxygenation capacity of stored RBCs. However, 2,3-DPG depletion appears to recover within a few hours post-transfusion.

Cold storage of RBCCs induces inactivation of membrane ionic pumps, as shown by Na^+ uptake and K^+ loss throughout the storage period[55]. Moreover, low-temperature storage slows metabolic enzyme activities, inducing a progressive decrease of ATP level[55]. D'Alessandro *et al*[56] further reported that accumulation of the metabolic intermediates NADPH and 6-phosphogluconate led to activation of the oxidative phase of the pentose phosphate pathway (PPP). NADPH is required for the reduction of oxidized glutathione (GSSG), recycling reduced glutathione (GSH) to counteract accumulating reactive oxygen species (ROS). However, GSH level has been shown to continuously decrease over the duration of RBC storage[53]. The literature includes contradictory descriptions of GSSG level, with some studies reporting an increase[56] and other showing a decrease with no concomitant activation of the PPP[57].

RBCs also have to deal with oxidative stress due to their constant exposure to oxygen fluctuations. Erythrocytes assist with maintaining circulatory antioxidant levels, particularly by recycling oxidized forms of plasma ascorbic acid[58], and contributing to the extracellular pool of GSH[59]. Despite their characteristic absence of mitochondria, RBCs can also act as ROS generators. Indeed, oxyhemoglobin dissociation to deoxyhemoglobin through oxygen release can promote iron electron capture by oxygen. Due to its ferric (Fe^{3+}) iron state, methemoglobin cannot bind oxygen, but the enzyme methemoglobin reductase can convert methemoglobin back to Fe^{2+} normal hemoglobin (Hb). This Hb autoxidation occurs at a rate of 3%, and leads to formation of the superoxide radical $O_2^{\bullet -}$[60], further producing the highly reactive hydroxyl radical $HO^{\bullet}$ through the Fenton reaction[61]. Such radicals are extremely reactive, possessing a very short half-life of approximately 10^{-9} seconds[62]. Hence, they can non-specifically affect all types of macromolecules within their immediate environment. This can lead to several types of RBC storage lesion, include lipid peroxidation, protein amino acids modifications, and protein backbone breaks[63,64].

The oxidative lesion is of particular interest, since such damage is not reversed through the transfusion process, in contrast to biochemical modifications. Since erythrocytes are unable to perform protein synthesis, no protein turnover is possible. Damaged proteins accumulate until they are degraded or eliminated from the cell. Redox proteomic studies performed on stored RBCs show increasing hallmarks of oxidative stress throughout the storage period. Several protein carbonylation investigations have indicated that increased storage duration is related to increased carbonylated protein contents associated with the RBC membrane and cytoskeleton[65-67], or with entire erythrocytes[53]. Blasi *et al*[53] further demonstrated that protein carbonylation was due to increasing oxidative stress concomitant with overactivation of the oxidative phase of the PPP, recruited to counteract ROS. Protein oxidation can also be investigated in terms of cysteine redox status, but such an approach has not yet been employed in the transfusion field. Oxidation reportedly results in

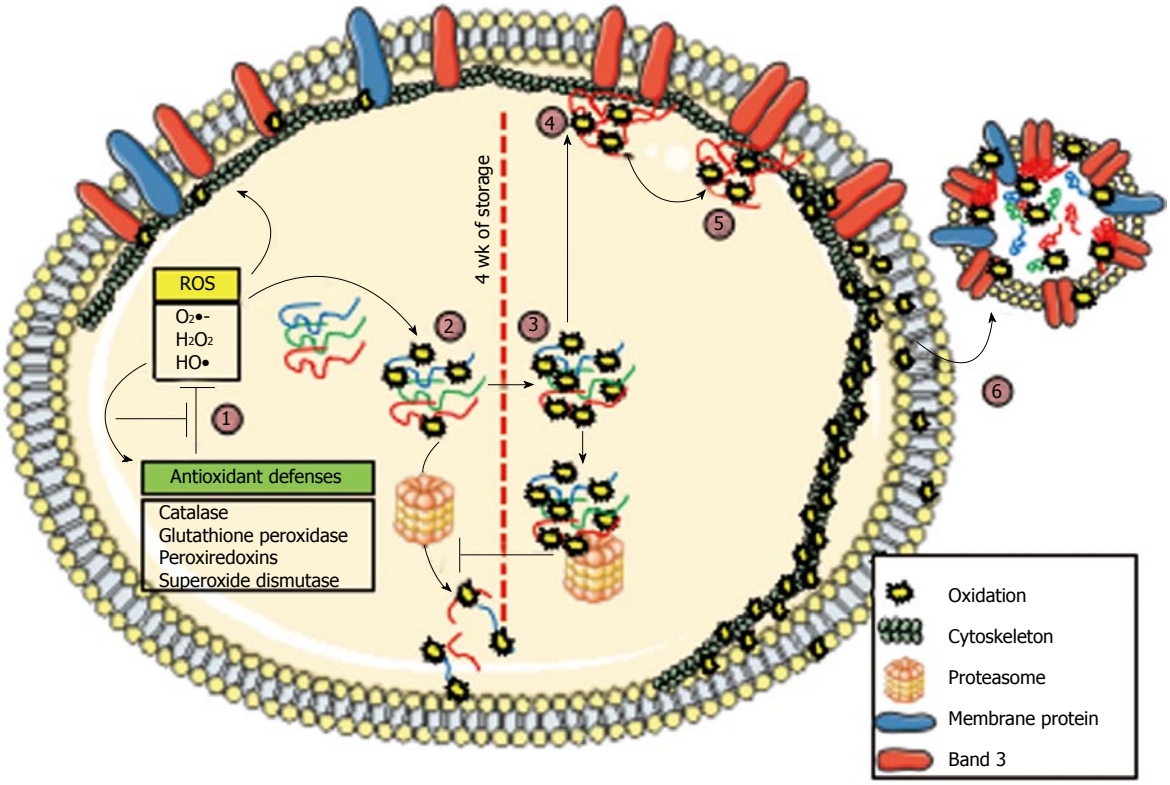

Figure 1 Representation of oxidative pathways involved in red blood cell storage. The model shows an red blood cell (RBC), divided in the middle (dashed line) to show features of early (left half) and late stages (right half) of oxidative damage. Membrane-bound and cytosolic components are indicated in the legend. A microvesicle is shown outside the RBC (right). Progressive oxidation steps are indicated as follows: Step 1: (Left of midline) RBC antioxidant defenses prevent most oxidative damage by reducing reactive oxygen species (ROS), which converts ROS to less-reactive intermediates, or by becoming oxidized by ROS, and then recycling through a restorative mechanism. After prolonged storage, these defenses are overwhelmed by oxidative stress and protein oxidation, which occurs in both the cytosol and the plasma membrane; Step 2: As oxidized proteins unfold, they expose hydrophobic moieties that are recognized by 20S proteasome complexes, which perform proteolysis. Antioxidant defense proteins are also oxidized, and they become susceptible to proteolysis; the lack of defense leads to the overoxidation of RBC content (right of midline); Step 3: At around 4 wk of storage, overoxidized proteins undergo crosslinking, which prevents further degradation; thus, partially-oxidized proteins and damaged proteins accumulate; Step 4: Oxidized hemoglobin aggregates into hemichromes, which bind to the membrane-bound band 3 protein; this binding modifies band 3 conformation, which potentially alters its association with the cytoskeleton, and the hemichromes displace the glycolytic enzymes bound to band 3; Step 5: Hemichrome autoxidation produces ROS, which oxidize cytoskeletal and membrane proteins; Step 6: The release of microvesicles enters an exponential phase, which allows the elimination of RBC aging markers, such as altered band 3 and externalized phosphatidylserine. Reprinted from ref. [65], with permission from Elsevier.

hemoglobin crosslinking, forming hemichromes that covalently bind the cytoplasmic domain of the major integral glycoprotein of the erythrocyte membrane (band 3), inducing its clustering[68-70]. The induced conformational change creates a neo-antigen site at the erythrocyte surface[71,72], allowing *in vivo* recognition by naturally occurring auto-antibodies[73-77]. In turn, this lead to RBC clearance from circulation through complement activation[78] and phagocytosis by Kupffer liver cells[79,80]. This is known as the band 3 clustering model.

Hemichromes are also capable of autoxidation, producing ROS in the membrane and cytoskeletal environment. Protein carbonylation, a hallmark of oxidative modifications, reportedly increases in the cytoskeletal protein population during RBC storage[67]. Such cytoskeletal alteration involves the third type of storage lesion: The biomechanical lesion. Indeed, rheological properties of RBCs apparently undergo alterations throughout the storage period, directly impacting the *in vivo* capacities of transfused RBCs. They become more rigid and thus less able to flow through the capillary micro-circulation, diminishing their tissue oxygenation power. Most remarkably, their shape is modified, evolving from a normal discoid shape to a spherocyte, with intermediate echinocyte and sphero-echinocyte shapes[81]. Along with this shape evolution, RBCs release increasing amounts of microvesicles (MVs)[82], and exhibit reduced deformability[83], a tendency to aggregate[81], enhanced adherence to endothelial cells[84,85], and increasing susceptibility to hemolysis[86]. The microvesiculation is believed to be a protective mechanism[87] based on observations of enriched altered band 3 (initiating RBC clearance) in MVs[24,88-90]. Figure 1 summarizes some of the biochemical events associated with the formation of MVs.

In an editorial recently published in *Blood Transfusion*, Piccin *et al*[91] discussed the importance of studying red blood cell microparticles. In the physiological state, phospholipid vesicles of less than 1 μm are present, and their concentrations may vary under pathological conditions. These vesicles generally originate from various types of cells, including red blood cells, platelets,

leukocytes, endothelial cells, and tumor cells. They participate in numerous biological processes (reviewed in[92-94]). Different types of these vesicles (EVs) have been described, notably exosomes (EXs) and microparticles (MPs), which are most likely generated *via* different mechanisms, although they share many similarities presenting a challenge regarding their purification and characterization. EXs are small (40 to 100 nm in diameter) spherical vesicles of endocytic origin that are secreted upon fusion of the limiting membrane of multivesicular bodies with the plasma membrane. MVs have been described in blood samples obtained from patients with many different diseases, as well as in cases of storage lesion from red blood cell preparations dedicated for transfusion[93,95].

Red blood cell-derived microvesicles (REVS) express blood group antigens[92], and are involved in thrombin generation, and thus in coagulations processes[96], as well as in inflammation[97]. Throughout the storage period, RBCs release increasing amounts of MVs[82], with intense exponential release starting at four weeks of storage. As seen in stored ECs, MVs are also encountered *in vivo* in RBC populations that are heterogeneous regarding the age of cells. It remains unknown whether all cells or only old cells participate in the MV release. Thus, it cannot yet be determined whether cytoskeleton destabilization through hemichrome binding prompts the release of MVs. It is likely that hemichrome binding to membranes[69] induces conformational changes of band 3, creating a surface neo-epitope[76] that is recognized by naturally occurring anti-band 3 auto-antibodies[71,74], favoring *in vivo* RBC clearance. Moreover, storage-released MVs are reportedly enriched in such altered band 3. Thus, it seems likely that hemichrome binding to RBC membranes occurs before the massive release of MVs. One can further hypothesize that hemichrome binding may play a role in signaling towards microvesiculation, perhaps through destabilization of the membrane-to-cytoskeleton association. Overall, microvesiculation appears to be a potential mechanism of defense.

ONGOING RESEARCH TO IMPROVE RBC TRANSFUSION EFFICACY AND SAFETY

In general, the questions regarding transfusion-related adverse outcomes due to aged blood products are not considered a major concern since most RBCCs (notably those of groups A and O) are transfused during the first half of storage, at a mean of 12 d after collection, which is below the 14-d threshold utilized in most published studies and in the European Directorate guide. However, this threshold has not been substantiated by solid and valid scientific studies and with clinical registered trials. Simple dichotomization of fresh *vs* old blood products has not proven to be helpful for assessing issues related to the duration of RBC storage prior to transfusion, particularly as the authors of such studies have not agreed on any standard time cut-off for categorizing blood as short-stored or long-stored. If future clinical studies determine such a cut-off time, it may guide changes in all transfusion practices. Future research should be devoted to better evaluating additive solutions, storage conditions, pre-transfusion practices, and even hospital policies (for example, to deliver safer products or patient-specific products, or to limit the needs for transfusion). Several groups are already developing new strategies for better RBC preservation, as well as implementing special storage conditions (*e.g.*, anaerobic storage) and original pre-transfusion practices, such as washing end-stored RBCs.

ADDITIVE SOLUTIONS

RBCC storage represents a substantial improvement in the field of blood transfusion. The development of solutions allowing erythrocyte preservation for up to six weeks has been a long and slow process. The additive solution that are currently used worldwide must be approved by local drug administrations and comply with their guidelines. Almost everywhere, RBCC storage in SAG-M is allowed for 42 d, as such cells are effective at delivering O_2 to patients' tissues and raising Hb levels. However, this is not at all satisfactory in terms of RBC physiology, since RBC can maintain functional capacity for 120 d *in vivo*. Thus, much research has focused on further improving the existing additive solutions, and to otherwise improve the quality of stored RBCCs.

In particular, in the early 2000s, the Hess group published four papers in four years, introducing experimental storage solutions (EAS) allowing effective RBC preservation for 9 wk in EAS-61[98], 10 wk in EAS-64[99], 11 wk in EAS-67[100], and 12 wk in EAS-76[101]. Their studies reported progressive changes to AS-1-derived EAS that progressively prolonged the RBC storage duration while maintaining a hemolysis rate below 1% and an *in vivo* 24 h recovery of greater than 75%. Compared with the frequently used SAGM and AS-1/3/5 solutions, the EAS was created with a lower salt concentration, higher adenine content (higher AS volume), and a more alkaline AS pH, which seemed to enable longer preservation of ATP and 2,3-DPG concentrations in RBCs. The main drawback of the designed EAS is its alkaline pH that favors dextrose caramelization during the heat sterilization process. Thus, the components must be autoclaved separately then aseptically mixed. Attempts to prolong RBCC storage must account for ease of use, as well as associated variations regarding stability in blood bags, hemolysis, and survival.

REJUVENATION

In addition to developing more efficient additive solutions, the rejuvenation of stored RBCs before transfusion could produce RBCCs of better quality. Rejuvesol™

is a solution comprising pyruvate, inosine, adenine, Na_2HPO_4, and NaH_2PO_4. Incubating RBCs in Rejuvesol™ for 1 h at 37 ℃, reactivates RBC metabolism allowing replenishment of depleted ATP and 2,3-DPG levels. Afterwards, the rejuvenated cells must be washed to remove excesses of inosine, which are potentially toxic to the recipient. Using this process, Meyer *et al*[102] showed efficient increases of ATP and 2,3-DPG in EC samples from RBCs stored in AS-1, AS-3, and AS-5 for 30, 42, 60, 80, 100, and 120 d. Although post-rejuvenation ATP and 2,3-DPG levels were lower in RBCs with longer storage duration, even extremely long-stored RBCs were reportedly capable of a metabolism restart[103]. Another study conducted by Koshkaryev *et al*[104] demonstrated that rejuvenation efficiently reversed the adherence of stored RBCs to endothelial cells, as well as lowered levels of membrane phosphatidyl serine (PS) exposure and intracellular Ca^{2+} and ROS. It is possible that the washing step will induce hemolysis in fragilized older RBCs, but the damaged cells can be selectively removed from the blood product prior to transfusion. Cognasse *et al*[105] showed that modulators of inflammation may also be eliminated according. Finally, Pallotta *et al*[106] have reported that the addition of antioxidants may prove useful in rejuvenation processes.

PRE-TRANSFUSION WASHING OF LONG-STORED RBCS

Washing of RBCCs has been prescribed by Sir John Dacie for patients suffering from paroxysmal nocturnal hemaglobinuria (Marchiafava-Micheli syndrome). The procedure has been also used in patients with IgA defiencency, and/or in patients presenting with recurrent severe allergic transfusion reactions such as anaphylaxis or severe urticarial reactions not prevented by pre-transfusion antihistamine and corticosteroid administration.

In a recent prospective randomized clinical trial, Cholette *et al*[107] investigated the washing of stored RBCs and platelets preceding transfusion to pediatric cardiopulmonary bypass surgery patients. This pre-transfusion practice obviously reduced inflammation markers. Pre-transfusion washing also showed a non-significant association with fewer transfused blood units, and decreased mortality. More recently, Bennett-Guerrero *et al*[108] compared different devices for washing long-stored RBCCs. In addition to removing accumulated storage-related compounds (potassium and lactate), they found that the washing procedure induced higher hemolysis and MV release. Free Hb and RBC-derived MVs are known to be scavengers of the vasodilator nitric oxide (NO), and are thus responsible for transfusion-induced impaired vascular function[109]. However, the washed and recovered RBCs did not seem to be more sensitive to physical stress-induced hemolysis, but rather showed less filtration-related hemolysis, presumably because older erythrocytes had already lysed during the washing process. With regards to the washing devices, the applied *g* force seemed to impact the quality of the final washed product[108]. Another focus of interest related to RBC washing is the varying amount of residual plasma in ECs. Depending on the specific processing of the blood product, RBC units can contain anywhere from a few to 100 mL of anticoagulated plasma. Weber *et al*[110] demonstrated that in cases where the donor has high-strength anti-HLA class Ⅱ antibodies, transfusion related acute lung injuries can be attributed to the reactive residual plasma. Although this additional procedure is time-consuming, washing RBCs has the advantage of cleaning the blood products prior to transfusion.

AN OXIDATIVE PATHWAY MODEL OF RBC STORAGE

In addition to storage lesion, oxidative alterations (particularly cysteine residue oxidation) are involved in multiple cellular processes through oxidation-reduction cycles. In the best known example, soluble alcohol oxidase (AO) is less prone to irreversible cysteine oxidation during storage, suggesting that its active site would be preserved, allowing RBCs to fight against ROS. However, by half-way through the RBC storage period, AO shows a higher carbonylation status that correlates with the doubled incidence of oxidation. The global quantitative decrease in soluble carbonylated proteins should be related to preserved AO activity up to the first two weeks of storage. Up to day 26, AO proteins appear to accumulate oxidative injuries through carbonylation. However, from day 26 to day 41, the proportion of this protein family is reduced. This decrease could be explained by the fact that protein oxidation induces recognition and degradation by 20S proteasome complexes. Increasing oxidation of AO defenses, without altering their active sites or making them prone to proteolysis, is likely to change AO enzymes into proteasome inhibitors, as supported by the reported protein oxidation dose-dependent inhibitory effect on P20S activity.

In summary, AO and other proteins accumulate oxidative injuries until around four weeks of RBC storage, making them recognizable by P20S complexes such that they are degraded or they inhibit proteasomal activity. Eventually, AO defenses decrease to the extent that they cannot counterbalance oxidants, allowing overoxidation of proteins that can no longer be taken over by the proteolysis machinery as enzymes from the PPP seem to be. Protein overoxidation induces aggregate formation, and the aggregated forms of Hb are termed hemichromes and bind to band 3 on its cytoplasmic domain. Band 3 proteins are the loci of membrane-to-cytoskeleton binding, and of multiprotein complexes. In particular, band 3 complexes contain proteins belonging to the glycolysis chain. Hemichrome

binding is likely to affect the structure of the cytoplasmic domain of band 3, thus displacing these proteins and potentially destabilizing the cytoskeleton and altering RBC glycolytic functions. Hemichromes like Hb are capable of autoxidation, which may contribute to the oxidative injuries that accumulate on cytoskeleton proteins, membrane proteins, and glycolysis and PPP enzymes. Hemichrome binding and further oxidation of the cytoskeleton and membrane environment could be a signal for high release of aging markers and other modified proteins by microvesiculation.

Several clinical studies on blood age-related transfusion-linked adverse outcomes, as well as research studies on RBCC storage lesion and altered metabolism, suggest that there is a 14-d storage limit after which RBCs are not optimally suitable for transfusion. In contrast, our results suggest that oxidative stress-linked alterations of stored RBCs produce a longer storage limit of four weeks. Transfusion distribution statistics from many hospitals show that half of RBCCs are transfused during the first two weeks of storage, and that around 90% are transfused during the first four weeks. This suggests that, in most cases, transfused RBCs are "healthy" enough to ensure the transfusion goal of increasing tissue oxygenation capacities. However, this conclusion could depend on the storage conditions, particularly the chosen additive solution, and the hospital policies regarding EC delivery. With the advancement of patient-specific medicine, the storage of blood products for transfusion might also eventually become recipient-dependent.

OXYGEN-FREE RBC STORAGE

In frozen and stored RBCs, oxidative stress challenges proteins involved in redox regulation, energy metabolism, and cytoskeleton organization. To deal with this issue, research has investigated the use of anaerobic RBC storage. Yoshida *et al*[111] have reported promising results regarding the maintenance of 2,3-DPG and ATP levels during anaerobic RBC storage, as well as diminished MVs release by these RBCs throughout the storage period. In their 2007 study, they applied six gas exchange cycles, achieving a 50-fold reduction of free oxygen concentrations in RBC units[111]. Though impractical for routine RBCC storage, this setup resulted in increased *in vitro* membrane stability, with reduced hemolysis and MV release rates compared to the conventional six weeks of storage in AS-3. After one week of storage, 2,3-DPG was completely depleted regardless of the oxygenation state. However, ATP preservation was better under anaerobic conditions, with the initial level conserved for up to 7 wk, and only a 15% decrease found after 10 wk, compared to the 64% decrease seen with aerobic storage. The authors attributed this improved ATP maintenance to an increased glycolytic flux with reduced activation of the pentose phosphate pathway due to the lower NADPH oxidation rate.

In a second paper, Yoshida *et al*[112] investigated the pH of different anticoagulant solutions, as well as the use of a rejuvenation process, with anaerobically stored RBCs. At any pH, both parameters were better maintained throughout the storage period under anaerobic conditions compared with aerobic storage. Under anaerobic conditions, pH impacted ATP and 2,3-DPG preservation. While the pH of AS showed no effect on hemolysis, they observed a tendency towards reduced microvesiculation under acidic anaerobic conditions[112]. After seven weeks of acidic anaerobic storage, a rejuvenation process enabled a 24 h recovery of 77.3% of RBCs after 10 wk of storage, with a low rate of hemolysis (0.35%). This 24 h recovery remained acceptable for transfusion after 12 wk of storage, providing that a second rejuvenation process was performed after 11 wk. The use of a novel AS (OFAS-3) produced similarly improved parameters at current maximal storage duration, and the potential to prolong storage to up to 9 wk[113].

In addition to these markers of RBC storage and *in vivo* recovery data, D'Amici *et al*[114] investigated early storage-induced membrane protein degradation and highlighted the role of oxygen depletion in reducing such oxidative damage to proteins. The absence of oxygen can boost glycolysis, but will result in skipping of the pentose phosphate pathway and potentially reduce the GSH defenses.

HOSPITAL POLICIES LEAD TO TRANSFUSION OF FEWER RBCCS

Many countries in Europe and North America are seeing reduced demand for blood transfusion services from hospitals and physicians. Several parameters have contributed to the decrease of the demand: (1) introduction of patient blood management; (2) progress in surgical measures; (3) better anesthesiology; (4) use of cell severs; and (5) implementation of guidelines aiming to clearly describe transfusional indications notably those published by the AABB (http://www.aabb.org/sa/clinical-practice-guidelines/Pages/default.aspx), the ISBT (http://www.isbtweb.org/working-parties/clinical-transfusion/7-red-cell-transfusion-triggers/) of the American Society of Hematology (www.hematology.org/.../Guidelines-Quality/.../527*).* However, the number of published guilines ius correlated to the degree of uncertainty of the practices. A Pubmed search (2015-08-21) yielded 559 items using ((guidelines) AND (red blood cell OR erythrocytes) AND transfusion) and with a search performed using (strategies) AND (red blood cell OR erythrocytes) AND transfusion.

The 2013 hemovigilance report from Swissmedic[115] highlighted a countrywide 6% decrease of RBCC demand. Between 2008 and 2013, this reduction increased to 10.9%. These data are in accordance with new global hospital policies regarding transfusion. For instance, several 2014 papers published in *Transfusion*-the journal launched by the American Association of

Blood Banks (AABB)-described the restrictive blood transfusion practices implemented in hospitals in the United States. A retrospective study from the Roger Williams Hospital of Providence (Rhode Island) also revealed significantly lowered transfusion rates over a 9-year intervention period, with many RBCC transfusions in non-bleeding cases being cancelled or reduced based on patients' Hb levels[116]. Interestingly, this decrease in RBCC transfusion was closely correlated with a decrease of mortality (r = 0.88). Similarly, Yerrabothala *et al*[117] from the Dartmouth-Hitchcock Medical Center of Lebanon (New Hampshire) reported a decreased number of transfused ECs in accordance with new local policies, without changes in the lengths of stay or mortality rates. In another example, Goodnough *et al*[118] at the Stanford Hospital and Clinics (California) conducted a retrospective study of RBC transfusions and patient outcomes before and after the establishment of a new transfusion policy based on patient Hb level. They reported significant decreases of mortality, length of stay, and 30-d readmission rates thanks to the induced decline of transfusion rates.

In addition to lowered needs for RBCCs, work is being done to artificially produce red blood cells from the culture of hematopoietic stem cells. Luc Douay[119] has produced some rather promising results in this field, which will most likely benefit patients presenting with complex alloimmunization. Cultured RBCs have proven to be functional regarding deformability, enzyme content, Hb capacity to fix and release molecular oxygen, and expression of blood group antigens. Furthermore, cultured RBCs appeared to survive *in vivo* in humans, exhibiting up to 63% survival at 26 d post-injection. These adaptations are reasonable, and *ex vivo* produced RBCs may eventually become a sustainable surrogate for donated blood. However, artificial blood will not be commercially available soon, and it appears that natural blood will continue to be transfused for a long time.

CONCLUDING REMARKS AND PERSPECTIVES

The impact of the storage duration of transfused blood products, particularly regarding RBCCs, is a currently highly debated issue in the transfusion medicine field. Several clinical studies report a tendency for transfusion of "old blood" to be associated with adverse outcomes in recipients. But the precise definition of "old blood" remains unclear. We cannot yet define a storage duration after which the transfusion of an RBCC would be undoubtedly harmful for the recipient despite suitable transfusion prognostics, *e.g*., storage hemolysis and *in vivo* 24 h recovery.

There are several obvious reasons why clinical studies cannot delimit any such single storage duration. First, an RBCC is not a globally defined blood component. Variations in many production system and processing parameters, from blood collection to RBCC delivery, may be quite different among different countries. In particular, the additive solutions used to preserve RBCs during their non-physiological cold storage differ from place to place. Although the formulations of diverse AS seem similar in term of components, some subtle changes appear to substantially impact RBC storage. Energy metabolism is a key factor in RBC maintenance, which appears to be differentially impacted depending on the AS. Another factor complicating the establishment of a storage limit for a guaranteed successful transfusion procedure is the recipient's health status. The same product may have different effects on patients suffering from different healthy conditions. Along the same lines, the same component may have different impacts on two patients with the same hematologic disorder if they are of different ages or different sexes. It is thus difficult to attribute clinical adverse outcomes to a given storage duration, while in fact all other factors influencing a given transfusion procedure are not identical. Most clinical investigations regarding RBCC storage duration focus on one particular clinical situation, with a possible bias because of the topics of interest. In this case, multivariate analyses seems highly desirable to detect evidence-if any exists-that the storage duration of a blood factor has a substantial impact on the ultimate patient outcome, having accounted for a myriad of confounding parameters.

Longer RBCC storage duration leads to greater lesion. Clinical studies have not yet conclusively identified a storage limit before which this lesion will not be hazardous for transfusion recipients. Such a discovery would necessitate a reconsidering of practices regarding blood supply processing and use. Ongoing investigations are exploring new ways to store blood products, and to avoid lesion occurrence. New additive solutions, pre-transfusion procedures, and special storage conditions are being investigated, with encouraging findings. Particularly promising results have been reported with the use of an oxygen-depleted storage environment and the addition of antioxidants to additive solutions. Further research efforts should be invested in such strategies-not necessarily aiming at prolonging storage duration based on erythrocyte stability during storage and after transfusion, but rather at understanding what happens in stored RBCCS. Such efforts could aid in attempts to prevent or correct cell lesion, to find new markers of blood product quality, and most certainly to provide physicians and patients with blood components of the highest quality and metabolic safety.

Finally, research efforts have largely concentrated on blood components. However, from a historical point of view, it is possible that this method was erroneous or less than optimal. We should remain open to the possibility of reintroducing arm-to-arm transfusion, and thus avoiding any lesion attributable to storage, or to otherwise altering the paradigm of transfusion medicine that considers blood components rather than human

blood.

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Treatment of Epstein-Barr virus-related hemophagocytic lymphohistiocytosis: Study protocol of a prospective pilot study

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Abstract

In this manuscript, a number of debatable issues related to the diagnosis and treatment of Epstein-Barr virus-related hemophagocytic lymphohistiocytosis (EBV-HLH) will be addressed. Considering the heterogeneous nature of EBV-HLH, diagnostic efforts are required to clarify the precise nature of the disease at diagnosis, the number of EBV genome copies in peripheral blood, and localization of the EBV genome in lymphoid cells (B, T, or natural killer cells). Although the majority of cases of EBV-HLH develop without evidence of immunodeficiency, some cases have been found to be associated with chronic active EBV infection, genetic diseases such as X-linked lymphoproliferative disease (XLP, type 1, or type 2), or familial HLH (FHL, types 2-5). Due to such background heterogeneity, the therapeutic results of EBV-HLH have also been found to vary. Patients have been found to respond to corticosteroids alone or an etoposide-containing regimen, whereas other patients require hematopoietic stem cell transplantation. Thus, decision-making for optimal treatment of EBV-HLH and its eventual outcome requires evaluation in consideration of the precise nature of the disease. A protocol for a pilot study on the treatment of patients with EBV-HLH is presented here.

Key words: Hemophagocytic lymphohistiocytosis; Epstein-Barr virus; Immune-chemotherapy; Rituximab; Hematopoietic stem cell transplantation

Core tip: Diagnosis of Epstein-Barr virus-related hemophagocytic lymphohistiocytosis (EBV-HLH) must fulfill both the evidence of EBV infection and the diagnostic criteria for HLH. EBV-HLH is heterogeneous. The majority of EBV-HLH occurs in apparently immunocompetent subjects, but some are associated with chronic active EBV infection status, X-linked lymphoproliferative disease or with familial HLH. Thus, treatment and outcome differ significantly depending on the underlying disease. To find out a most appropriate treatment, various laboratory tests are required to clarify the underlying diseases.

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INTRODUCTION

Epstein-Barr virus-related hemophagocytic lymphohistiocytosis (EBV-HLH) is defined as a hemophagocytic syndrome associated with systemic EBV-related T cell or natural killer (NK) cell lymphoproliferative diseases (LPDs)[1]. There are two main types of HLH, primary (genetic, inherited) and secondary (acquired)[2]. EBV-HLH is heterogeneous; the majority of cases of EBV-HLH tend to occur in apparently immunocompetent subjects as secondary disease. However, a number of cases of EBV-HLH have been found to be associated with primary diseases such as familial HLH (FHL)[3,4] or with X-linked lymphoproliferative disease (XLP, type 1, or type 2)[5,6]. Recently, various types of primary immunodeficiencies were found to correlate with EBV-proliferative disease[7]. In EBV-HLH, EBV resides primarily in T cells or NK cells, and is more frequent in Asia and rarely in Western countries[3,8,9]. The outcome of EBV-HLH has been observed to significantly differ depending on the underlying diseases. Patients with secondary EBV-HLH have been found to respond well to immunotherapy or to chemoimmunotherapy, while patients with EBV-HLH associated with primary diseases require hematopoietic stem cell transplantation (HSCT) due to refractoriness to chemoimmunotherapy[3]. Thus, genetic studies on FHL and XLP[2,7,10] are essential to identify whether the patient has a primary condition. In addition, a poor outcome of the patients with chronic active EBV infection (CAEBV)-related HLH has been well recognized in Asia, particularly in Japan[11]. Although CAEBV has occasionally been reported in Western countries[12,13], the difference between CAEBV in Asia and Western countries remains elusive. It was reported that in Asia, EBV-infected T cell or NK cells have been found to play a major role in CAEBV, while in Western countries CAEBV primarily involves EBV-infected B cells[11,12]. In terms of EBV tropism in EBV-HLH, Kasahara and colleagues have demonstrated $CD8^+$ T cells to play a major role in acute onset EBV-HLH following initial EBV infection, whereas in CAEBV-HLH, involvement of $CD4^+$ T cells or NK cells were primarily found[14]. The findings that the outcome of CAEBV-related HLH is poor, but the genetic abnormalities of CAEBV have not been identified[3,14,15] suggest the importance of the identification of major cell types in patients with EBV-HLH upon diagnosis. In addition, in some patients with CAEBV-related HLH that have chromosome abnormalities of EBV-infected cells, a very poor outcome has been reported[16]. Thus, determination of karyotypes in the peripheral blood, bone marrow, or biopsied tissue for the prediction of the outcome of patients with EBV-HLH is also desirable. Lastly, viremia is quantitatively identified by determining the EBV genome copy numbers in peripheral blood. Genome copies are obtained per ml of serum/plasma, or per 10^6 cells (or μg DNA); however, the former is more commonly employed. Responsiveness or refractoriness of EBV-HLH against treatment can be evaluated by determining viral genome copy numbers[17,18]. Treatment of EBV-HLH has been found to be effective based on the HLH-94 and HLH-2004 type protocols[19,20] and has been confirmed on a global scale[21,22]. However, this type of treatment is not required for all cases of EBV-HLH[23,24]. In acute EBV-HLH, approximately 40% of patients may respond to prednisolone, cyclosporine, or intravenous immunoglobulin (IVIG) treatment, while 60% of patients require an etoposide-containing regimen[24]. However, details of the results of etoposide therapy, such as the exact duration of treatment or the total dose of etoposide administered to the patients, are unknown. As the HLH-94 and HLH-2004 protocols were originally proposed for primary (familial) HLH[19,20], the most appropriate treatment for patients with secondary EBV-HLH remains unknown.

DIAGNOSIS OF EBV-HLH

For diagnosis, the diagnostic criteria for HLH[20] must be fulfilled. Furthermore, EBV involvement must be verified by a positive anti-VCA-IgM (primary infection or reactivation), viremia (> 10^3 genome copies/mL of serum or plasma), or a positive EBER-ISH of the bone marrow clot or biopsied tissue section[3].

UNDERLYING DISEASES ASSOCIATED WITH EBV-HLH

EBV-HLH is a heterogeneous disease and occurs as an acute onset disease upon initial exposure to EBV, or in association with CAEBV, peripheral T cell lymphoma and NK cell leukemia or lymphoma, or a genetic disorder. Genetic disorders associated with EBV-HLH have been identified in patients with XLP (type 1 or type 2), FHL (types 2-5), or other rare genetic diseases[2,4-7,19] (Figure 1). Diagnostic criteria for CAEBV have been previously proposed[25]. Acute onset EBV-HLH has been defined as the development of HLH following initial exposure to EBV, while CAEBV-related HLH has been defined as the development of HLH during CAEBV (Figure 2).

TREATMENT RESPONSE CRITERIA IN EBV-HLH

Response to treatment has been defined as follows: initial good response (GR) has been defined as a complete resolution of fever and reduced serum ferritin values (< 500 ng/mL); complete response (CR) has

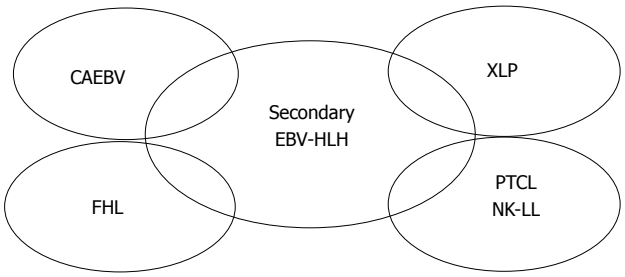

Figure 1 Underlying or other diseases overlapping with Epstein-Barr virus-related hemophagocytic lymphohistiocytosis. Although the majority of cases of EBV-HLH due to secondary HLH develop without any apparent immunodeficiency, some cases may develop in association with CAEBV (see also Figure 2), XLP (type 1 or type 2), FHL (types 2-5), or EBV-positive peripheral T cell lymphoma, or NK cell leukemia or lymphoma. EBV-HLH: Epstein-Barr virus-related hemophagocytic lymphohistiocytosis; CAEBV: Chronic active EBV infection; XLP: X-linked lymphoproliferative disease; FHL: Familial HLH; NK: Natural killer.

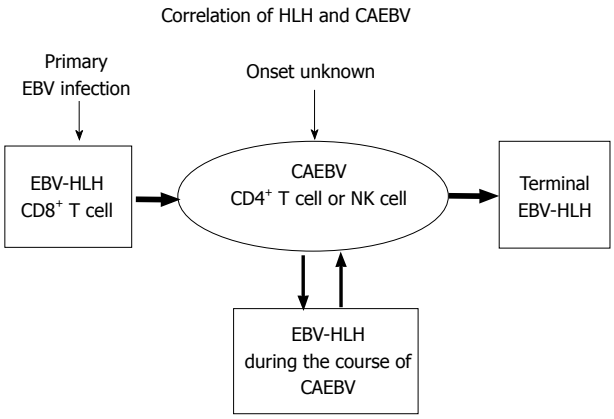

Figure 2 Correlations between hemophagocytic lymphohistiocytosis and chronic active Epstein-Barr virus infection status. CAEBV status may occur without apparent onset of symptoms or may develop following initial acute onset EBV-HLH. During the course of CAEBV, HLH episodes may develop, and if it is not adequately treated by transplantation, most patients eventually succumb to terminal HLH or to lymphoid malignancies. CD8+ T cells play a major role in initial acute onset HLH, whereas $CD4^+$ T cells or NK cells play a role in the status of CAEBV and in CAEBV-related HLH. CAEBV: Chronic active EBV infection; EBV-HLH: Epstein-Barr virus-related hemophagocytic lymphohistiocytosis; NK: Natural killer.

been defined as a complete resolution of fever, serum ferritin levels, and EBV genome copies in peripheral blood; and poor response (PR) has been defined as having no reduction of fever and continued high serum ferritin levels (> 500 ng/mL). Relapse has been defined as the recurrence of fever associated with both increased serum ferritin levels and EBV genome copies in peripheral blood. Refractory disease has been defined as the presence of active disease following completion of treatment[26].

PROPOSED PILOT PROTOCOL

Diagnosis of EBV-HLH is achieved by performing flow cytometry of peripheral blood, quantification of EBV genome copy numbers, and serological detection of anti-EBV-VCA-IgM. The proposed pilot protocol is depicted in Figure 3. Treatment consists of a window period of 2 wk treated with prednisolone (PSL, A1; 2 mg/kg per day). If a GR is attained, PSL with tapering (A2) is further given. At the end of 3 wk of A2 arm treatment, no more treatment is given for patients who attained a CR. Patients with a PR/NR to A1 arm go to B regimen, which consists of weekly etoposide (100 mg/m^2), PSL (2 mg/kg) and cyclosporine A (CSA; trough levels 80-150)[27]. If EBV resides in B cells, 3 doses of rituximab (375 mg/m^2 per dose) could be added in B regimen[28-30]. Patients not attaining a CR at the end of B arm treatment go further to C regimen, which consists of once every 2 wk of etoposide (100 mg/m^2), PSL (2 mg/kg) and cyclosporine A (CSA; trough levels 80-150). If the patient attains a CR at the end of 24 wk from onset of treatment becomes off treatment and a total cumulative dose of etoposide will be 2200 mg/m^2. Patients remain at NR/PR at the end of 24 wk of treatment go to a salvage therapy or to HSCT. During the initial 8 wk of treatment, EBV-HLH should be characterized as either acute onset EBV-HLH, CAEBV-related HLH, XLP-related HLH, or FHL-related HLH. Patients demonstrating progressive disease in association with XLP, FHL, or CAEBV should be considered for HSCT as early as possible. Ideally, quantification of the EBV genome copy number should be performed at the onset of treatment, following 4, 8, 24, 28, 72, and 96 wk of treatment, and up to 2 years post-treatment, as indicated in the protocol.

Primary aims of the pilot study

The pilot study treatment protocol will explore the following questions concerning EBV-HLH: (1) what are the major cell types at the onset of EBV-HLH: B cells, $CD4^+$ T cells, $CD8^+$ T cells, or NK cells; (2) how is the CAEBV status initiated? A hypothesis has been previously proposed[26]. The majority of cases of CAEBV have been suggested to occur insidiously without apparent onset of symptoms, but some cases may develop following initial acute onset EBV-HLH (Figure 2); (3) how high is the incidence (%) of CAEBV-related or genetic disease-related HLH among refractory cases of EBV-HLH; (4) how high is the incidence (%) of patients treated with PSL alone among the CR group? How many patients (%) require treatment using the HLH-94 or HLH-2004 type regimen? What is the correlation between disease type (B cell *vs* T or NK cell, acute onset *vs* others) and treatment response; (5) how do changes in EBV genome copy numbers correlate with treatment response; (6) how do changes in serum ferritin levels correlate with clinical symptoms and EBV genome copy numbers; (7) how many doses of etoposide when necessary are required to attain a CR? When can treatment be discontinued in patients with secondary EBV-HLH; (8) how many cumulative doses of etoposide can be safely administered without causing therapy-related acute myeloid leukemia (t-AML); and (9) how does EBV-HLH differ between Asian and Western countries?

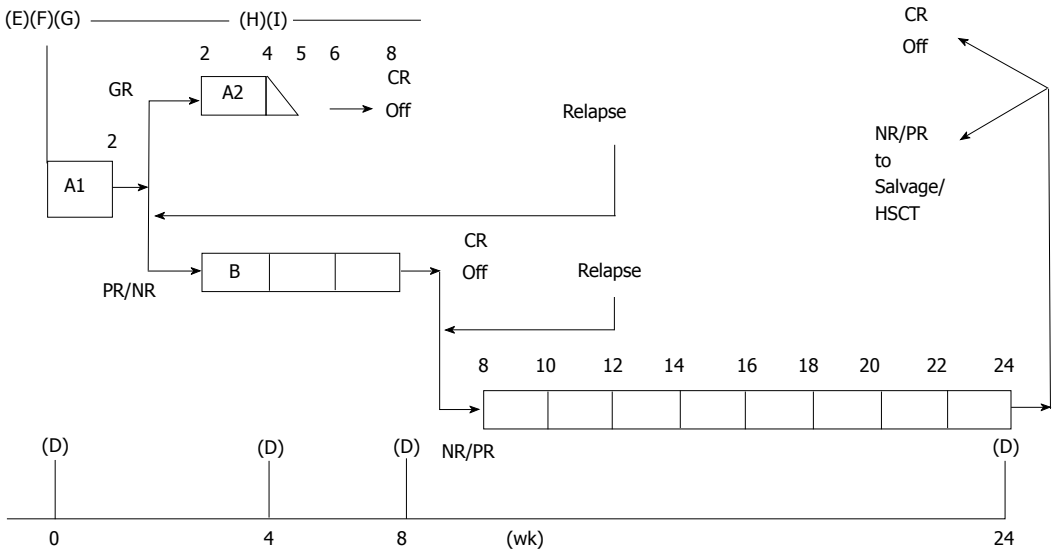

Figure 3 Treatment regimens for Epstein-Barr virus-related hemophagocytic lymphohistiocytosis. Prior to commencement of treatment, determination of EBV genome copy numbers (D), EBV serology (anti-VCA-IgM, -IgG,-EADR-IgG and anti-EBNA) (E), flow cytometry (F), and, if possible, EBV tropism in subsets (G) of PB are required. Furthermore, cytogenetics (H) of PB or bone marrow cells are recommended. It is also recommended to determine EBV genome copy numbers (D) following 4, 8, and 24 wk of treatment to observe treatment response, and following 24 wk, 12, 18, and 24 mo of treatment to determine whether the disease progresses to the status of CAEBV. Screening tests for XLP or FHL are ideally required (I) for any cases demonstrating a PR/NR to the A1 and B regimens until 8 wk of treatment. Treatment comprises a window period of 2 wk commencing with PSL (A1; 2 mg/kg per day). Once a GR is attained, PSL with tapering (A2) is administered. Following 5 wk of PSL given, treatment is discontinued in patients who attain a CR. Patients with a PR/NR to A1 and those relapsed with A2 are to commence the B regimen, which comprises a weekly dose of etoposide (100 mg/m^2), PSL (2 mg/kg), and CSA (trough levels, 80-150). If EBV is found to reside in B cells, three doses of rituximab (375 mg/m^2 per dose) are then added to the B regimen. Patients that do attain a CR becomes off therapy at the end of 8 wk, while who do not attain a CR with B arm treatment are to commence the C regimen, which comprises a once every 2 wk dose of etoposide (100 mg/m^2), PSL (2 mg/kg), and CSA (trough levels, 80-150). Patients who relapse after CR with B arm also go to C regimen. If the patient attains a CR following 24 wk of treatment, the total cumulative dose of etoposide is 2200 mg/m^2. Patients that remain at PR/NR following a total 24 wk of treatment are to undergo salvage therapy or HSCT. GR: Good response; PR: Poor response; NR: No response; CR: Complete response; HSCT: Hematopoietic stem cell transplantation; PSL: Prednisolone; CSA: Cyclosphorin A.

DISCUSSION

EBV-LPDs and EBV-HLH

EBV-HLH is a hemophagocytic syndrome occurring in patients with EBV-associated LPDs. The development of EBV-LPD is linked to various hereditary or acquired immune deficiencies[7], such as XLP1, XLP2, interleukin-2-inducible T cell kinase deficiency, CD27 deficiency, or XMEN (X-linked immunodeficiency with Mg^{2+} defect, EBV infection and neoplasm) syndrome, or may occur post-transplantation. In a study comprising 108 patients with EBV-associated T/NK cell LPDs in Japan, 80 patients were found to have CAEBV, 15 patients were found to have acute onset HLH, nine patients were found to have a severe mosquito bite allergy, and four patients were found to have hydroa vacciniforme[11]. It remains unclear how HLH occurs in patients with EBV-LPDs. Although EBV-HLH is significantly prevalent in Asia, its incidence and characteristics in Western countries not only require clarification but also a comparison with those observed in Asia.

Heterogeneity of EBV-HLH

Although EBV-HLH may primarily appear to be a secondary disease, it is rather heterogeneous, including high risk diseases such as CAEBV and hereditary diseases. In our previous study comprising 94 EBV-HLH patients, 60 patients were found to be anti-VCA-IgM-positive, indicating that no primary infectious EBV serological patterns were detected in approximately one third of the patients[3]. To determine if EBV is found to reside in B cells, $CD4^+$ T cells, $CD8^+$ T cells, or NK cells, assessment of viral tropism is recommended to be performed at the time of diagnosis, *via* cell sorting and quantification of EBV genome copy counts in each lymphocyte subset. Alternatively, flow cytometry of peripheral blood may be performed to determine the major cell types involved in EBV-HLH (Figure 2).

Quantification of EBV genome copy numbers

The diagnosis of EBV-HLH may be possible using serology alone in cases of a positive anti-VCA-IgM, although determination of the degree of viremia over time is far more useful to assess the response and determine a correlation with the status of CAEBV. Thus, quantifying EBV genome copy numbers in peripheral blood at the onset of HLH and during the course of treatment is highly recommended[17,18]. Clinically sensitive markers to evaluate EBV-HLH activity include fever, high serum ferritin levels, lactate dehydrogenase, soluble interleukin-2 receptor, and high EBV genome copy numbers. In our previous study, patients with persistently high EBV genome copy numbers in peripheral blood eventually required HSCT due to refractory disease[18]. However, we recently observed a number of patients who were symptomless and in a

stable condition following discontinuation of treatment for EBV-HLH despite persistent viremia (Unpublished observations). Long-term follow-up may reveal whether these patients relapse as HLH or eventually attain a CAEBV status. There is no consensus on how to best treat these patients.

Acute onset EBV-HLH and CAEBV-related HLH

Previous reports of EBV-HLH potentially comprised a mixture of secondary acute onset HLH, CAEBV-related, or primary/genetic disease-related HLH[3,31]. In addition to genetic diseases, the CAEBV status should be taken into account in cases of EBV-HLH. Patients with CAEBV are accompanied with dermal symptoms defined as hypersensitivity to mosquito bites or hydroa vacciniforme, and development of HLH in association with clinical features such as infectious mononucleosis-like symptoms, lymphadenopathy, adult onset Still's disease-like symptoms, cardiovascular complications, or cerebellar ataxia or encephalitis[11,32-34]. In EBV-HLH, classification of secondary HLH as either occurring upon initial exposure to EBV or due to reactivation in association with CAEBV is essential, as the outcome significantly differs between the two[15,24]. We have previously observed approximately 85% of patients with EBV-HLH to be treated with immunochemotherapy alone compared with 15% who required HSCT[31]. Patients who underwent HSCT were most likely to comprise high risk groups such as CAEBV-related HLH, XLP-related HLH, or FHL-related HLH.

Treatment of EBV-HLH

In the treatment of EBV-HLH, administration of an etoposide-based regimen within 4 wk of diagnosis has been found to have a beneficial effect[27], although several studies also have reported a CR in a subset of patients using conventional PSL, CSA, or IVIG alone[23,24]. The proposed study herein may clarify the true nature of the disease and whether treatment with etoposide is necessary. Furthermore, treatment with etoposide should be commenced immediately following 2 wk of prednisolone therapy in cases where PR/NR is observed.

Cumulative doses of etoposide and t-AML

The use of etoposide may be of concern to physicians due to the patients' risk of developing t-AML. In my survey, twelve documented cases of t-AML in HLH have been reported in the literature. (Unpublished observations) The median occurrence of t-AML following HLH treatment has been found to be 26 mo (range, 6 mo to 6 years). Of these 12 cases, the 11q23 abnormality was found in four cases, FAB-M3 leukemia was observed in two cases, FAB-M5 leukemia was found in two cases, and other types of leukemia were observed in four cases. In seven patients, cumulative doses of etoposide were observed to be greater than 3000 mg/m^2, and doses less than 1500 mg/m^2 were found in five cases. Based on these data, to reduce the incidence of t-AML in HLH treated patients, cumulative doses of etoposide should be preferably limited to less than 3000 mg/m^2. In the proposed pilot protocol presented here, upon completion of treatment in patients who undergo the arm B regimen within 8 wk, the total etoposide dose comprises 600 mg/m^2. Even if the patients undergo the arm B and C regimens, the total dose comprises 2200 mg/m^2, which is considered to be in a safe range.

Rituximab treatment

Rituximab is a pre-emptive B-cell-directed therapy and a candidate for the treatment of EBV-HLH in which EBV resides within B cells[28-30]. However, in the majority of EBV-HLH cases, EBV resides in T cells or in NK cells. As one of the mechanisms behind why T cells or NK cells that lack a receptor for EBV are infected with the virus, EBV-infected B cells are hypothesized to potentially transfer the virus to T cells or NK cells due to contact between EBV-infected B cells and cytotoxic T cells or NK cells. In consideration of this hypothesis, rituximab may even be effective in the treatment of EBV-HLH involving T cells or NK cells[35]. Whether rituximab administration is applicable for all cases of EBV-HLH remains to be explored. As a salvage therapy, alemtuzumab[36] and other regimens may also be used. Novel chemo-therapeutic agents for the treatment of CAEBV are currently in progress[37]. It remains unknown if salvage therapy alone may provide a cure for refractory EBV-HLH.

Adoptive cell therapy

Adoptive immunotherapy has been shown to be effective in the treatment of CAEBV[38,39]. Similar adoptive cell therapies are expected to be effective in refractory EBV-HLH. Based on fetal-maternal microchimerism tolerance, Wang and colleagues infused high doses of HLA-haploidentical maternal peripheral blood mononuclear cells (> 10^8/kg per infusion) in five patients with EBV-positive T cell-LPD, with CR observed in three patients[40].

HSCT

In patients with CAEBV-related or genetic disease-related HLH, allogeneic HSCT is essential, although one of the biggest challenges is the timing of the transplant. The overall estimated 3-year survival outcome after HSCT has been found to be 62% (± 12%) in patients with FHL[19]. In 2008, Sato and co-workers surveyed 74 cases of HSCT in EBV-associated T cell or NK cell-LPDs in Japan that comprised 42 cases of CAEBV, ten cases of EBV-HLH, and 22 cases of EBV-associated leukemia or lymphoma. In the study, the event-free survival rate was found to be 0.561 ± 0.086 for CAEBV, 0.614 ± 0.186 for EBV-HLH, and 0.309 ± 0.107 for EBV-lymphoma or leukemia[41]. In 2010, Ohga *et al*[42] analyzed the outcomes of HSCT on 43 FHL and 14 EBV-HLH patients, in which the 10-year overall survival rates were found to be 65.0% ± 7.9% in FHL and 85.7% ± 9.4% in EBV-

HLH patients. Another retrospective study has been performed on CAEBV patients (n = 17) who underwent HSCT with a reduced intensified conditioning regimen (RIC) followed by bone marrow transplantation (RIC-BMT), and in patients (n = 15) who underwent RIC followed by unrelated cord blood transplantation (RIC-UCBT). Excellent overall survival rates were obtained with RIC-BMT (92.9% ± 6.9%) and RIC-CBT (93.3% ± 6.4%) (P = 0.87)[43]. In a more recent study, all five CAEBV patients who underwent HSCT have been reported to be alive without any serious regimen-related toxicity for more than 16 mo following HSCT[44]. In consideration of these results, HSCT may be safely performed in patients to obtain a cure for refractory EBV-HLH or CAEBV. In addition, the efficacy of UCBT in combination with the RIC regimen has been confirmed in the treatment of EBV-HLH and CAEBV. However, decision-making concerning the determination of the optimal time to perform transplantation at a particular stage of the disease is often difficult in patients that remain in a stable condition, although HSCT may be a curable measure for CAEBV-related HLH and other hereditary disease-related EBV-HLH.

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