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and treatment of polycythemia vera**

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Lek XOSPATA

Wskazany w monoterapii nawrotowej lub odpornej na leczenie ostrej białaczki szpikowej z mutacją FLT3 u dorosłych pacjentów i stosowany doustnie, pozwala uzyskać trwałą supresję mutacji FLT3, jednocześnie oferując wygodę leczenia w warunkach domowych¹



Rozpoznanie

Nawrotowa AML z obecnością mutacji FLT3-TKD

Lucyna, 57 lat

XOSPATA Nawrót Dobry stan ogólny

dla dorosłych pacjentów w dobrym stanie ogólnym z R/R FLT3m+ AML, takich jak Lucyna¹



Rozpoznanie

Oporna na leczenie AML z obecnością mutacji FLT3-ITD

Józef, 67 lat

XOSPATA Oporność Dobry stan ogólny

dla dorosłych pacjentów w dobrym stanie ogólnym z R/R FLT3m+ AML, takich jak Józef¹



Rozpoznanie

Nawrotowa AML z mutacjami FLT3-ITD i IDH2 oraz zidentyfikowanie odpowiedniego dawcy do HSCT

Paweł, 43 lata

XOSPATA Nawrót Dobry stan ogólny

dla dorosłych pacjentów w dobrym stanie ogólnym z R/R FLT3m+ AML, takich jak Paweł¹



Rozpoznanie

Nawrotowa AML z mutacjami FLT3-ITD, NPM1 i IDH1

Joanna, 77 lat

XOSPATA Nawrót Gorszy stan ogólny

dla dorosłych pacjentów w gorszym stanie ogólnym z R/R FLT3m+ AML, takich jak Joanna¹

XOSPATA może pomóc pacjentom w dobrym i gorszym stanie ogólnym, takim jak Lucyna, Józef, Paweł i Joanna, w osiągnięciu dłuższego czasu przeżycia¹

Lucyna, Józef, Paweł i Joanna nie są prawdziwymi pacjentami.

1. Charakterystyka Produktu Leczniczego XOSPATA.

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Nazwa produktu leczniczego: Xospata 40 mg, tabletki powlekane. **Skład jakościowy i ilościowy:** Każda tabletka powlekana zawiera 40 mg gilteritynibu (w postaci fumaranu). Pełny wykaz substancji pomocniczych, patrz punkt 6.1 Charakterystyki Produktu Leczniczego (ChPL). **Postać farmaceutyczna:** Tabletka powlekana (tabletki). **Wskazania do stosowania:** Produkt Xospata jest wskazany w monoterapii nawrotowej lub opernej na leczenie ostrej białaczki szpikowej (ang. *acute myeloid leukaemia*, AML) z mutacją *FLT3* u dorosłych pacjentów (patrz punkty 4.2 i 5.1 ChPL). **Dawkowanie i sposób podawania:** Leczenie produktem Xospata powinien rozpocząć i nadzorować lekarz mający doświadczenie w stosowaniu terapii przeciwnowotworowych. Przed przyjęciem gilteritynibu u pacjentów z nawrotową lub operną na leczenie AML należy potwierdzić mutację FMS-podobnej kinazy tyrozynowej 3 (ang. *FMS-like tyrosine kinase 3*, *FLT3*) [wewnątrznaczadnikowa duplikacja genu (ang. *internal tandem duplication*, ITD) lub mutację w obrębie domeny kinazy tyrozynowej (ang. *tyrosine kinase domain*, TKD)] przy użyciu zwalidowanego testu. Podawanie produktu Xospata można wznowić u pacjentów po przeszczeniu krwiotwórczych komórek macierzystych (ang. *haematopoietic stem cell transplantation*, HSCT). **Dawkowanie:** Zalecana dawka początkowa to 120 mg gilteritynibu (trzy tabletki po 40 mg) raz na dobę. Przed rozpoczęciem leczenia, w 15. dniu, a następnie co miesiąc przez cały czas trwania leczenia należy ocenić badania biochemiczne krwi, w tym aktywność fosfokinazy kreatynowej (patrz punkt 4.4 ChPL). Przed rozpoczęciem leczenia gilteritynibem, w 8. i 15. dniu pierwszego cyklu oraz przed rozpoczęciem każdego kolejnego miesiąca leczenia przez następne trzy miesiące należy wykonać badanie elektrokardiograficzne (EKG) (patrz punkty 4.4 i 4.8 ChPL). Kobiętom w wieku rozrodczym należy doradzić wykonanie testu ciążowego w ciągu siedmiu dni przed rozpoczęciem leczenia produktem leczniczym Xospata (patrz punkt 4.4, i 4.6 ChPL). Leczenie należy kontynuować do czasu, kiedy pacjent nie będzie już odnosił korzyści klinicznych z leczenia produktem Xospata lub do momentu wystąpienia niedopuszczalnej toksyczności. Odpowiedź na leczenie może być opóźniona, dlatego należy rozważyć kontynuowanie stosowania zalecanej dawki do 6 miesięcy, aby zapewnić czas na odpowiedź kliniczną. W przypadku braku odpowiedzi (pacjent nie osiągnął złożonej całkowitej remisji (CRc)) po 4 tygodniach leczenia można zwiększyć dawkę do 200 mg (pięć tabletek po 40 mg) raz na dobę, jeżeli leczenie jest tolerowane lub uzasadnione klinicznie. **Modifikacje dawki:** Zalecenia dotyczące przerwy w podawaniu, zmniejszenia dawki i zaprzestania podawania produktu Xospata u pacjentów z nawrotową lub operną na leczenie AML: **Zespół różnicowania:** W przypadku podejrzenia zespołu różnicowania podać kortykosteroidy i rozpocząć monitorowanie hemodynamiczne (patrz punkt 4.4 ChPL). **Przerwać stosowanie gilteritynibu**, jeśli ciężkie objawy przedmiotowe i/lub podmiotowe utrzymują się przez ponad 48 godzin po rozpoczęciu stosowania kortykosteroidów. **Wznowić leczenie gilteritynibem**, podając taką samą dawkę, gdy nasilenie objawów przedmiotowych i podmiotowych zmniejszy się do stopnia 2° lub niższego. **Zespół odwracalnej tylniej encefalopatii:** Zaprzestać stosowania gilteritynibu. **Odstęp QTcF > 500 ms:** Przerwać stosowanie gilteritynibu. **Wznowić leczenie gilteritynibem**, podając zmniejszoną dawkę (80 mg lub 120 mg*), gdy odstęp QTcF powróci do wartości w zakresie 30 ms wartości początkowej lub ≤ 480 ms. **Zwiększenie odstępu QTcF o więcej niż 30 ms w badaniu EKG w 8. dniu pierwszego cyklu:** Potwierdzić w badaniu EKG w 9. dniu. W przypadku potwierdzenia należy rozważyć zmniejszenie dawki do 80 mg. **Zapalenie trzustki:** Przerwać podawanie gilteritynibu aż do ustąpienia zapalenia trzustki. **Wznowić leczenie gilteritynibem**, podając zmniejszoną dawkę (80 mg lub 120 mg*). **Inna toksyczność stopnia 3° lub wyższego uznawana za związaną z leczeniem:** Przerwać stosowanie gilteritynibu aż do ustąpienia toksyczności lub zmniejszenia jej nasilenia do stopnia 1°. **Wznowić leczenie gilteritynibem**, podając zmniejszoną dawkę (80 mg lub 120 mg*). **Planowane HSCT:** Przerwać stosowanie gilteritynibu na jeden tydzień przed zastosowaniem leczenia kondycjonującego w HSCT. Leczenie można wznowić 30 dni po HSCT, jeśli nastąpiło wszczepienie, u pacjenta nie wystąpiła ostra postać choroby przeszczep przeciw gospodarzowi (stopień ≥ 2), i znajdował się on w złożonej całkowitej remisji (CRc): *a. Stopień 1. oznacza nasilenie „łagodne”, stopień 2. oznacza nasilenie „umiarkowane”, stopień 3. oznacza nasilenie „ciężkie”, stopień 4. oznacza nasilenie „zagrożające życiu”.* *b. Dawka dobową może być zmniejszona ze 120 mg do 80 mg lub z 200 mg do 120 mg.* *c. CRc jest definiowana jako współczynnik remisji we wszystkich całkowitych remisji (definicja całkowitej remisji, patrz punkt 5.1 ChPL), CRp [całkowita remisja z niepełną regeneracją płytek krwi (< 100 x 10⁹/l)] i CRi [spełnione kryteria całkowitej remisji z wyjątkiem pełnej regeneracji hematologicznej, z utrzymującą się neutropenią < 1 x 10⁹/l i całkowitą regeneracją płytek krwi lub bez niej]. Osoby w podeszłym wieku:* Nie jest wymagane dostosowanie dawki u pacjentów w wieku ≥ 65 lat (patrz punkt 5.2 ChPL). **Zaburzenia czynności wątroby:** Nie ma konieczności dostosowania dawki u pacjentów z łagodnymi (klasa A wg skali Child-Pugh) lub umiarkowanymi (klasa B wg skali Child-Pugh) zaburzeniami czynności wątroby. Produktu Xospata nie zaleca się do stosowania u pacjentów z ciężkimi zaburzeniami czynności wątroby (klasa C wg skali Child-Pugh), ponieważ w tej populacji nie oceniano bezpieczeństwa stosowania i skuteczności (patrz punkt 5.2 ChPL). **Zaburzenia czynności nerek:** Nie ma konieczności dostosowania dawki u pacjentów z zaburzeniami z zaburzeniami umiarkowanymi lub ciężkimi (patrz punkty 4.4 i 5.2 ChPL). **Dzieci i młodzież:** Nie określono dotychczas bezpieczeństwa stosowania ani skuteczności produktu leczniczego Xospata u dzieci w wieku poniżej 18 lat. Dane nie są dostępne. Ze względu na wiązanie się z receptorem 5HT_{2B} w warunkach *in vitro* (patrz punkt 4.5 ChPL) u pacjentów w wieku mniej niż 6 miesięcy istnieje możliwość oddziaływania na rozwój serca. **Sposób podawania:** Produkt Xospata jest przeznaczony do podania doustnego. Tabletki można przyjmować z posiłkiem lub bez. Należy je połkać w całości, popijając wodą i nie należy ich przełamywać ani rozkruszać. Produkt Xospata należy podawać mniej więcej o tej samej porze każdego dnia. Jeśli dawka zostanie pominięta lub nie zostanie przyjęta o zwykłej porze, należy podać dawkę jak najwcześniej tego samego dnia, a pacjent powinien powrócić do zwykłego schematu dawkowania następnego dnia. Jeśli podaniu dawki wystąpią wymioty, pacjenci nie powinni przyjmować kolejnej dawki, tylko powinni powrócić do zwykłego schematu dawkowania następnego dnia. **Przedwskazania:** Nadwrażliwość na substancję czynną lub na którąkolwiek substancję pomocniczą wymienioną w punkcie 6.1 ChPL. **Specjalne ostrzeżenia i środki ostrożności dotyczące stosowania:** **Zespół różnicowania:** Stosowanie gilteritynibu wiązało się z występowaniem zespołu różnicowania (patrz punkt 4.8 ChPL). Zespół różnicowania polega na szybkiej proliferacji i różnicowaniu komórek mielodajnych. Nieleczony może zagrażać życiu lub prowadzić do zgonu. Objawy podmiotowe i stan kliniczny w zespole różnicowania obejmują gorączkę, duszność, wysięk opłucynowy, wysięk osierdziowy, obrzęk płuc, niedociśnienie tętnicze, szybki przyrost masy ciała, obrzęk obwodowy, wysypkę i zaburzenia czynności nerek. W przypadku podejrzenia zespołu różnicowania należy rozpocząć leczenie kortykosteroidami wraz z monitorowaniem hemodynamicznym aż do ustąpienia objawów podmiotowych. Jeśli ciężkie objawy przedmiotowe i/lub podmiotowe utrzymują się przez ponad 48 godzin po rozpoczęciu stosowania kortykosteroidów, należy przerwać stosowanie gilteritynibu do czasu ustąpienia ciężkiego nasilenia objawów przedmiotowych i podmiotowych (patrz punkty 4.2 i 4.8 ChPL). Dawkę kortykosteroidów można zmniejszyć po ustąpieniu objawów podmiotowych i podawać je przez minimum 3 dni. Przedwczesne zakończenie leczenia kortykosteroidami może spowodować nawrót podmiotowych objawów zespołu różnicowania. **Zespół odwracalnej tylniej encefalopatii:** Zgłaszano występowanie zespołu odwracalnej tylniej encefalopatii (ang. *posterior reversible encephalopathy syndrome*, PRES) u pacjentów otrzymujących gilteritynib (patrz punkt 4.8 ChPL). PRES to rzadkie, odwracalne zaburzenie neurologiczne, które może manifestować się szybko rozwijającymi się objawami podmiotowymi obejmującymi drgawki, ból głowy, stan splątania, zaburzenia widzenia i zaburzenia neurologiczne z towarzyszącym nadciśnieniem tętniczym i zaburzeniami stanu psychicznego lub bez nich. W przypadku podejrzenia PRES należy to potwierdzić radiologicznym badaniem obrazowym mózgu, najlepiej metodą rezonansu magnetycznego (ang. *magnetic resonance imaging*, MRI). Zaleca się zaprzestanie leczenia gilteritynibem u pacjentów, u których wystąpił PRES (patrz punkty 4.2 i 4.8 ChPL). **Wydłużony odstę QT:** Stosowanie gilteritynibu wiązało się z wydłużeniem czasu repolarizacji komór serca (odstęp QT) (patrz punkty 4.8 i 5.1 ChPL). Wydłużenie odstępu QT można zaobserwować w pierwszych trzech miesiącach leczenia gilteritynibem. Dlatego też przed rozpoczęciem leczenia, w 8. i 15. dniu pierwszego cyklu i przed rozpoczęciem każdego kolejnego miesiąca leczenia, przez następne trzy miesiące należy wykonać badanie elektrokardiograficzne (EKG). Należy zachować ostrożność u pacjentów z istotnym wywiadem kardiologicznym. Hipokaliemia lub hipomagnezemia mogą zwiększać ryzyko wydłużenia odstępu QT. Dlatego też przed rozpoczęciem leczenia gilteritynibem i w jego trakcie należy wyrównać hipokaliemię lub hipomagnezemię. Należy przerwać stosowanie gilteritynibu u pacjentów, u których odstę QTcF > 500 ms (patrz punkt 4.2 ChPL). Decyzję o wznowieniu leczenia gilteritynibem po wystąpieniu wydłużenia odstępu QT należy podjąć po dokładnej ocenie korzyści i ryzyka. Jeżeli stosowanie gilteritynibu uznawia się w zmniejszonej dawce, po 15 dniach dawkowania i przed rozpoczęciem każdego kolejnego miesiąca leczenia, przez następne trzy miesiące należy wykonać badanie EKG. W badaniach klinicznych 12 pacjentów miało odstę QTcF > 500 ms. Trzech pacjentów przerwało i ponownie rozpoczęło leczenie bez nawrotu wydłużenia odstępu QT. **Zapalenie trzustki:** Zgłaszano przypadki zapalenia trzustki. Należy badać i monitorować pacjentów, u których wystąpiły objawy przedmiotowe i podmiotowe sugerujące zapalenie trzustki. Należy przerwać stosowanie gilteritynibu, ale można je wznowić, podając zmniejszoną dawkę, gdy ustąpią objawy przedmiotowe i podmiotowe zapalenia trzustki (patrz punkt 4.2 ChPL). **Ciężka niewydolność nerek:** Narażenie na gilteritynib może być zwiększone u pacjentów z ciężkimi zaburzeniami czynności nerek lub szybką niewydolnością nerek. Podczas stosowania gilteritynibu należy ściśle monitorować pacjenta pod kątem toksyczności (patrz punkt 5.2 ChPL). **Interakcje:** Jednoczesne podawanie leków indukujących CYP3A4/P-gp może prowadzić do zmniejszonej ekspozycji na gilteritynib i, w konsekwencji, ryzyka braku skuteczności. Dlatego należy unikać jednoczesnego stosowania gilteritynibu z silnymi induktorami CYP3A4/P-gp (patrz punkt 4.5 ChPL). Należy zachować ostrożność w przypadku jednoczesnego przepisywania gilteritynibu z produktami leczniczymi, które są silnymi inhibitorami CYP3A, P-gp i/lub białka oporności na kaskiersi (ang. *breast cancer resistant protein*, BCRP), ponieważ mogą one zwiększać ekspozycję na gilteritynib. Należy rozważyć zastosowanie alternatywnych produktów leczniczych, które nie hamują silnie aktywności CYP3A, P-gp i/lub BCRP. W sytuacjach, gdy nie istnieją zadowalające alternatywy terapeutyczne, pacjentów należy ściśle monitorować pod kątem wystąpienia toksyczności w trakcie podawania gilteritynibu (patrz punkt 4.5 ChPL). Gilteritynib może zmniejszyć działanie produktów leczniczych, dla których receptorem docelowym jest receptor 5HT_{2B} lub nieswoiste receptory sigma. Dlatego też należy unikać równoczesnego stosowania gilteritynibu z tymi produktami, chyba że jego stosowanie uznaje się za niezbędne dla pacjenta (patrz punkt 4.5 ChPL). **Działanie toksyczne na zarodek lub płód i antykoncepcja:** Należy poinformować kobiety w ciąży o potencjalnym ryzyku dla płodu (patrz punkty 4.6 i 5.3 ChPL). Kobiętom w wieku rozrodczym należy doradzić wykonanie testu ciążowego w ciągu siedmiu dni przed rozpoczęciem leczenia gilteritynibem i stosowanie skutecznej metody antykoncepcji w trakcie leczenia gilteritynibem i przez co najmniej 6 miesięcy od zakończenia leczenia. Kobiety stosujące antykoncepcję hormonalną powinny dodatkowo stosować antykoncepcję barierową. Mężczyznom, których partnerki są w wieku rozrodczym, należy doradzić stosowanie skutecznej metody antykoncepcji w trakcie leczenia i przez co najmniej 4 miesiące od przyjęcia ostatniej dawki gilteritynibu. **Działania niepożądane:** Podsumowanie profilu bezpieczeństwa: Bezpieczeństwo produktu Xospata oceniono u 319 pacjentów z nawrotową lub operną na leczenie AML, którzy otrzymali co najmniej jedną dawkę 120 mg gilteritynibu. Najczęstszymi działaniami niepożądanymi gilteritynibu były zwiększenie aktywności aminotransferazy alaninowej (ang. *alanine aminotransferase*, ALT) (82,1%), zwiększenie aktywności aminotransferazy asparaginowej (ang. *aspartate aminotransferase*, AST) (80,6%), zwiększenie aktywności fosfatazy alkalicznej we krwi (68,7%), zwiększenie aktywności kinazy kreatynowej we krwi (53,9%), biegunka (35,1%), zmęczenie (30,4%), nudności (29,8%), zaparcia (28,2%), kaszel (28,2%), obrzęki obwodowe (24,1%), duszność (24,1%), zawroty głowy (20,4%), niedociśnienie tętnicze (17,2%), ból kończyn (14,7%), astenia (13,8%), ból stawów (12,5%) i ból mięśni (12,5%). Najczęściej ciężkimi działaniami niepożądanymi były ostre uszkodzenie nerek (6,6%), biegunka (4,7%), zwiększenie aktywności ALAT (4,7%), duszność (3,4%), zwiększenie aktywności AspAT (3,1%) i niedociśnienie tętnicze (2,8%). Inne, klinicznie istotne, ciężkie działania niepożądane obejmowały zespół różnicowania (2,2%), wydłużenie odstępu QT na elektrokardiogramie (0,9%) i zespół odwracalnej tylniej encefalopatii (0,6%). Działania niepożądane zaobserwowane w trakcie badań klinicznych wymieniono poniżej zgodnie z klasyfikacją układów i narządów MedDRA i według kategorii częstości. Kategorie częstości są zdefiniowane następująco: bardzo często (≥ 1/10), często (≥ 1/100 do < 1/10), niezbyt często (≥ 1/1000 do < 1/100), rzadko (≥ 1/10000 do < 1/1000), bardzo rzadko (< 1/10000) i częstość nieznaną (nie może być określona na podstawie dostępnych danych). W obrębie każdej grupy częstości występowania działań niepożądanych przedstawiono według zmniejszającej się ciężkości. **Zaburzenia układu immunologicznego:** Często: reakcja anafilaktyczna (1,3**; 1,3***). **Zaburzenia układu nerwowego:** Bardzo często: zawroty głowy (20,4**; 0,3***). Niezbyt często: zespół odwracalnej tylniej encefalopatii (0,6**; 0,6***). **Zaburzenia serca:** Często: wydłużenie odstępu QT na elektrokardiogramie (8,8**; 2,5***), wysięk osierdziowy (4,1**; 0,9***), zapalenie osierdzia (1,6**; 0,***), niewydolność serca (1,3**; 1,3***). **Zaburzenia naczyniowe:** Bardzo często: niedociśnienie tętnicze (17,2**; 7,2***). **Zaburzenia układu oddechowego, klatki piersiowej i śródpiersia:** Bardzo często: kaszel (28,2**; 0,3***), duszność (24,1**; 4,4***). Często: zespół różnicowania (3,4**; 2,2***). **Zaburzenia żołądka i jelit:** Bardzo często: biegunka (35,1**; 4,4***), nudności (29,8**; 1,9***), zaparcia (28,2**; 0,6***). **Zaburzenia wątroby i dróg żółciowych:** Bardzo często: zwiększenie aktywności aminotransferazy alaninowej* (82,1**; 12,9***), zwiększenie aktywności aminotransferazy asparaginowej* (80,6**; 10,3***). **Zaburzenia mięśniowo-szkieletowe i tkanki łączne:** Bardzo często: zwiększenie aktywności fosfokinazy kreatynowej we krwi* (53,9**; 6,3***), zwiększenie aktywności fosfatazy alkalicznej we krwi* (68,7**; 1,6***), ból kończyn (14,7**; 0,6***), ból stawów (12,5**; 1,3***), ból mięśni (12,5**; 0,3***). Często: ból mięśniowo-szkieletowy (4,1**; 0,3***). **Zaburzenia nerek i dróg moczowych:** Często: ostre uszkodzenie nerek (6,6**; 2,2***). **Zaburzenia ogólne i stany w miejscu podania:** Bardzo często: zmęczenie (30,4**; 3,3***), obrzęki obwodowe (24,1**; 0,3***), astenia (13,8**; 2,5***). Często: złamopodobałość (4,4**; 0,***). * Częstość opiera się na wartościach laboratoryjnych centralnego. ** Wszystkie stopnie ciężkości (%). *** Stopnie ciężkości ≥ 3 (%). **Opis wybranych działań niepożądanych: Zespół różnicowania:** W badaniach klinicznych z udziałem 319 pacjentów leczonych produktem Xospata u 11 (3%) wystąpił zespół różnicowania. Zespół różnicowania polega na szybkiej proliferacji i różnicowaniu komórek szpiku i nieleczony może zagrażać życiu lub prowadzić do zgonu. Objawy podmiotowe i stan kliniczny w zespole różnicowania u pacjentów leczonych produktem Xospata obejmowały gorączkę, duszność, wysięk opłucynowy, wysięk osierdziowy, obrzęk płuc, niedociśnienie tętnicze, szybki przyrost masy ciała, obrzęk obwodowy, wysypkę i zaburzenie czynności nerek. W niektórych przypadkach jednocześnie wystąpiła ostra gorączkowa dreszczowa neutrofilia. Zespół różnicowania wystąpił od jednego dnia (najwcześniej) do 82 dni od rozpoczęcia leczenia produktem Xospata i przebiegał ze współistniejącą leukocytosą lub bez niej. Z 11 pacjentów, u których wystąpił zespół różnicowania, 9 (82%) powróciło do zdrowia po okresie leczenia lub po przerwie w dawkowaniu produktu Xospata. Zalecenia w przypadku podejrzanego zespołu różnicowania podano w punktach 4.2 i 4.4 ChPL. **PRES:** W badaniach klinicznych z udziałem 319 pacjentów leczonych produktem Xospata u 0,6% wystąpił zespół odwracalnej tylniej encefalopatii (PRES). PRES to rzadkie, przemijające zaburzenie neurologiczne, które może objawiać się szybko rozwijającymi się objawami podmiotowymi obejmującymi drgawki, ból głowy, stan splątania, zaburzenia widzenia i zaburzenia neurologiczne z towarzyszącym nadciśnieniem tętniczym lub bez niego. Objawy podmiotowe ustąpiły po zaprzestaniu leczenia (patrz punkty 4.2 i 4.4 ChPL). **Wydłużenie odstępu QT:** Spośród 317 pacjentów leczonych produktem leczniczym Xospata w dawce 120 mg w badaniach klinicznych, którym zmierzono QTc po rozpoczęciu badania (ang. *post-baseline*), u 4 pacjentów (1%) stwierdzono odstę QTcF > 500 ms. Ponadto, w zakresie wszystkich dawek, u 12 pacjentów (2,3%) z nawrotową lub operną na leczenie AML maksymalna wartość odstępu QTcF po rozpoczęciu badania (ang. *post-baseline*) wynosiła > 500 ms (patrz punkty 4.2, 4.4 i 5.1 ChPL). **Zgłaszanie podejrzanego działania niepożądanego:** Po dopuszczeniu produktu leczniczego do obrotu istotne jest zgłaszanie podejrzanego działania niepożądanego. Umożliwia to nieprzerwaną monitorowanie stosunku korzyści do ryzyka stosowania produktu leczniczego. Osoby należące do fachowego personelu medycznego powinny zgłaszać wszelkie podejrzanego działania niepożądane za pośrednictwem Departamentu Monitorowania Niepożądanych Działań Produktów Leczniczych Urzędu Rejestracji Produktów Leczniczych, Wyrobów Medycznych i Produktów Biobójczych, Al. Jerozolimskie 181C, PL-02 222 Warszawa, tel.: +48 22 4921 301, faks: +48 22 4921 309, strona internetowa: <https://smz.ezdrowie.gov.pl>. **Podmiot odpowiedzialny:** Astellas Pharma Europe B.V., Sylviushweg 62, 2333 BE Leiden, Holandia. **Numery pozwoleń na dopuszczenie do obrotu:** EU/1/19/1399/001 – wydane przez Komisję Europejską. **Kategoria dostępności:** Produkt leczniczy wydawany na receptę do zastrzeżonego stosowania – Rpz.

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A – akalabrutynib. BTKi – inhibitor kinazy tyrozynowej Brutona. Clb – chlorambucyl. O – obinutuzumab.

1. Calquence-H-C-005299-X-0009-G: EPAR – Assessment report, EMA/38803/2023. 2. Sharman J.P. et al. Leukemia. 2022. doi:10.1038/s41375-021-01485-x. 3. Byrd J.C. et al. J. Clin. Oncol. 2021. https://doi.org/10.1200/JCO.21.01210.

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EFS – przeżycie wolne od zdarzeń; SOCT – standardowe leczenie

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Mutation status of immunoglobulin variable heavy chain genes (*IGHV*) – from immunological curiosity to forefront of diagnostics in chronic lymphocytic leukaemia

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B lymphocytes, by producing antibodies, are responsible for one of the two major arms of the immune response, the humoral one. In order to catch up with the constantly changing repertoire of pathogens and their new structures, B cells developed during evolution unique mechanisms involving manipulation with their genes to generate billions of receptors from a limited set of genes. Some of these mechanisms are shared with T lymphocytes, such as V-D-J gene segment rearrangement, while some are unique to B cells such as hypermutation of immunoglobulin genes. The latter mechanism occurs in the peripheral lymphatic organs upon antigen stimulation.

It has been exactly 25 years since two seminal papers published in 1999 in *Blood* by Damle et al. and Hamblin et al. [1, 2] clearly indicated that the mutational status of *IGHV* (immunoglobulin variable heavy chain genes) does matter in the biology of chronic lymphocytic leukaemia, a B-cell derived neoplasm, and represents an independent prognostic factor. In the accompanying editorial Naylor and Capra wrote: “most clinical laboratories do not have the capacity to isolate and characterise IgV gene sequences” [3].

We have come a long way since then, and fortunately this test has become the standard for all patients diagnosed with CLL. However, determination of *IGHV* status still remains a challenge for many labs, even those with

long experience in genetic diagnostics of haematological malignancies.

In this issue of *Acta Haematologica Polonica*, Zawada et al. [4] address comprehensively the current methodology and reporting guidelines for *IGHV* mutational status in CLL. This was an eagerly awaited publication. While several laboratories in Poland have recently earned an ERIC (European Research Initiative on CLL) society certificate (or are in the process of acquiring one), many issues remain problematic. There are doubts regarding methodology and how to deal with problematic samples.

We have now two major techniques: one that employs ‘old’ Sanger sequencing, and the second being NGS (next-generation sequencing). The authors explain in detail both methods, presenting their pros and cons. Importantly, guidelines for reporting are carefully prepared and discussed, including for problematic cases.

The only topic which has not been addressed by Zawada et al. is the financial challenge, this problem now being a major one for most laboratories, namely how to carry out all the molecular and cytogenetic tests that are now mandatory in CLL, within the current financial limits.

However, this is probably a topic for a separate article, especially given that this challenge is by no means CLL-specific.

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Recommendations of Polish Adult Leukemia Group concerning diagnostics and treatment of polycythemia vera

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Abstract

Polycythemia vera (PV) belongs to a group of myeloproliferative neoplasms (MPN). The diagnosis of PV is based on the evaluation of complete blood count, bone marrow trephine biopsy samples, and molecular tests (confirmation of the JAK2 mutation).

The main objectives of PV treatment include preventing thromboembolic complications, alleviating disease symptoms, and lowering the risk of transformation to myelofibrosis, acute myeloid leukemia and myelodysplastic syndrome. Taking into consideration the long-term course of PV, the choice of therapy should be based on an analysis of the risks resulting from the disease as well as from the adverse effects of the medication applied. In patients without risk factors (age <60 years; no history of thrombosis), acetylsalicylic acid (ASA) and phlebotomy are recommended. Cyto-reductive treatment is recommended in patients with poor tolerance of phlebotomy and with leukocytosis >20 G/L, symptomatic progressive splenomegaly, significant thrombocytosis, persistent general symptoms of PV, and also in patients with significant cardiovascular risk.

The therapy of choice in these patients is interferon α (IFN α). In a high-risk group of patients, (age $\geq$ 60 years and/or a history of thrombosis), treatment with ASA and phlebotomy should be accompanied by cytoreduction: hydroxyurea (HU) or, in the case of HU resistance/intolerance, ruxolitinib (RUX) or IFN α .

This paper discusses some aspects of the risk evaluation and summarizes the effectiveness of the available therapies, and also presents diagnostic and therapeutic recommendations for PV.

Keywords: polycythemia vera, myeloproliferative neoplasms, diagnosis, treatment

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Introduction

According to the International Consensus Classification (ICC) and to the 2022 classification of the World Health Organization (WHO), polycythemia vera (PV) is included in the group of myeloproliferative neoplasms (MPN), which are neoplasms of the hematopoietic stem cell [1, 2]. On the basis of population studies, the incidence rate of PV is estimated to be c. 1–1.5/100,000 people per year, with median age at onset 61 years, and with comparable incidence in both sexes [3, 4]. In the pathogenesis of the disease, the key role is played by the overactivity of the JAK-STAT (Janus kinase/signal transducers and activators of transcription) signaling pathway and the clonal hyperplasia of cells of bone marrow origin, resulting in the overproduction of blood morphotic elements, most expressed in the erythroid line. The diagnosis of the disease is based on assessment of the peripheral blood, molecular analysis, and histological assessment of a trephine bone marrow sample. The most significant characteristic of PV is polyglobulia. Additionally, approximately half of the patients have thrombocytosis and leukocytosis, and approximately one third have spleen enlargement [5]. The bone marrow abnormalities involve a typical picture of proliferation of all blood hematopoietic lines (panmyelosis) [1, 2]. A mutation in exon 14 of the *JAK2* gene (*JAK2* Val617Phe), discovered in 2005, is responsible for the development of the disease in 97% of patients with PV [6–8]. In other cases, mutations within exon 12 of the *JAK2* gene or non-canonical mutations are found [9–11]. Both at the moment of diagnosis and within the course of the disease, patients may be asymptomatic or present symptoms resulting from increased blood viscosity and from microcirculation disorders. In the majority of PV patients, the course of disease takes many years. It has been proven, however, that patients' predicted lifespan is shorter than in the case of healthy people of the same age, in particular among the younger patient population [12]. The main cause of morbidity and mortality in patients with PV comprises thromboembolic complications, both of venous and arterial types. Moreover, as a result of chronic, increased cell proliferation in the bone marrow and overproduction of proinflammatory cytokines, some of the patients may experience disease progression to post-polycythemia vera myelofibrosis (PPV-MF). Where there is accumulation of secondary cytogenetic and molecular events, inhibition of differentiation may occur with the evolution of the disease phenotype to the form resembling acute myeloid leukemia (AML), which is associated with a significantly worse prognosis [5].

Due to the phenotypically diverse patient population and the long-term course of the disease, PV therapy must be conducted based on the analysis of the risk of complications resulting from both the disease itself and the expected prolonged exposure to the therapy used. The objective of

the treatment is, first of all, the prevention of thromboembolic complications, alleviation of disease symptoms, and the decrease of the risk of transformation to PPV-MF, AML or myelodysplastic syndrome (MDS).

This article discusses some aspects of the risk evaluation and summarizes the effectiveness of the available therapies, and also presents diagnostic and therapeutic recommendations for PV.

Prognostic factors

Factors affecting risk of progression and survival time

In an international study carried out on 1,545 subjects with PV, the independent risk factors for overall survival (OS) were: leukocytosis >15 G/L, venous thrombosis and defective karyotype [5]. The negative impact of persistent leukocytosis on disease progression was also proven in another recent study [13]. The risk of progression of PV to MF and AML within 10 years is estimated to be 4.9–6% for MF and 2–5% for AML. After 20 years, these risks are estimated to be 26% and below 10% respectively [14]. The risk factors of leukemia transformation comprise: old age, defective karyotype, leukocytosis ≥ 15 G/L, and exposure to such medications as pipobroman, radioactive phosphorus (P^{32}), chlorambucil, but not hydroxyurea (HU) or busulfan [14]. The risk factors of MF transformation include the allele burden of *JAK2*V617F exceeding 50%, the presence of bone marrow fibrosis at the moment of diagnosing the condition, and persistent leukocytosis [13, 15–17]. Some recent studies have confirmed an adverse effect on OS caused by defective karyotype, leukocytosis, and some mutations not linked with *JAK2*, such as *SRSF2* and *IDH2* [18, 19]. The Mutation-Enhanced International Prognostic Scoring System for Polycythemia Vera (MIPSS-PV) prognoses OS, and the risk of MF or AML transformation, on the basis of demographic, clinical and genetic data [19]. This model was created on the basis of analysis of 404 PV patients, and takes into consideration a negative role of unfavorable spliceosome *SRSF2* mutation, age above 67 years, leukocytosis ≥ 15 G/L, and a history of a thromboembolic incident [19]. This model is presented in Table I.

Next generation sequencing (NGS) suggests that more than 50% patients with PV have other variants or mutations than those linked with the *JAK2* gene, with the mutations most frequently occurring in the *TET2* (18%), *ASXL1* (15%) and *LNK* (3%) genes [19, 20]. The combined incidence of unfavorable mutations (*SRSF2*, *IDH2*, *RUNX1*, *U2AF1*) was estimated to be c.15%. These mutations were shown to be negative risk factors for OS, leukemia-free survival or progression-free survival to myelofibrosis [19]. Additional prognostic information obtained from NGS or karyotype testing turns out to be very useful, but is not mandatory in routine clinical practice. Additional studies are definitely

Table I. Risk stratification for overall survival (OS) in polycythemia vera on basis of MIPSS-PV model ([19]).

MIPSS-PV	
A history of thrombosis	1 point
WBC ≥ 15 G/L	1 point
Age > 67 years	2 points
SRSF2 mutation	3 points
Risk stratification and interpretation	
Low risk	0–1 point; mOS 24 years
Medium risk	2–3 points; mOS 13.1 years
High risk	≥ 4 points; mOS 3.2 years

MIPSS-PV – Molecular International Prognostic Scoring System for PV; mOS – median overall survival; WBC – white blood cells

needed to confirm the reliability of the MIPSS-PV model, and to identify specific cytogenetic aberrations with prognostic significance [21, 22].

Stratification of thrombotic risk

The traditional stratification of thrombotic risk in PV distinguishes between two categories of risk: high (age > 60 years or a history of thrombosis) and low (the absence of both risk factors) [23]. The occurrence of arterial or venous thrombosis is the most important risk factor for subsequent vascular events [23]. However, it should be stressed that patients classified as low-risk have a significantly higher rate of thromboembolic complications compared to subjects of the same age without PV. Therefore, it is necessary to revise the definition of high risk in PV [24]. Patients with a mean hematocrit (Hct) value of $\geq 45\%$ compared to patients with Hct $< 45\%$ have a significantly higher risk of vascular events and death (hazard ratio [HR] 3.91, 95% CI 1.45–10.53, $p = 0.007$) [25]. Some recent studies have also identified hyperlipidemia and diabetes mellitus as risk factors for arterial events, and leukocytosis > 11 G/L and major bleeding at diagnosis (defined as gastrointestinal, internal organ, intra-articular, cerebrovascular, retroperitoneal or any bleeding requiring medical or surgical intervention, hospitalization or leading to death) as risk factors for venous thrombosis [26]. However, a systematic review and meta-analysis of articles involving more than 30,000 patients suggest that leukocytosis above the upper limit of normal plays an important role here in increasing the incidence of arterial but not venous thrombotic incidents [27]. The negative effect of hypertension on arterial thrombosis was demonstrated in another study [28], which listed individual cardiovascular risk factors important in predicting thrombotic events; and also indicated the need to optimize acetylsalicylic acid (ASA) dosage, but the evidence was not strong enough to distinguish the risk of thrombosis in the PV patients from the same risk in the general population [29, 30].

For this reason, the European Society of Cardiology risk classification has been adopted as a reference for assessing baseline vascular risk in PV patients [31, 32]. A calculator for the risk assessment is available at www.heartscore.org [33]. In patients who have already experienced their first thrombotic event, risk factors for recurrence include age > 60 years as well as leukocytosis at the time of the first event of arterial thrombosis in patients below 60 [34]. Additional factors that increase the risk of thrombosis include, in addition to leukocytosis, *JAK2V617F* allele burden $> 50\%$ and the frequency of phlebotomy [15, 27, 30, 34–37].

Diagnostics

The basic abnormalities in peripheral blood counts in which PV is suspected comprise increased Hct and elevated hemoglobin (Hgb) level. According to the 2022 WHO criteria, an Hgb concentration of more than 16.5 g/dL in men and 16.0 g/dL in women or Hct $> 49\%$ in men and $> 48\%$ in women is necessary for the diagnosis of PV [1]. More than 50% of patients have an increased platelet count and c.40% of patients have an increased leukocyte count (mainly neutrophils, but basophilia may also occur) [1]. The diagnosis of PV is based on close correlation of clinical, molecular and histological data. The diagnostic criteria developed by the WHO include blood morphology parameters, bone marrow histology, the presence of *JAK2* mutations, and decreased endogenous erythropoietin (Epo) levels [1]. The diagnosis of PV requires the fulfillment of all three major criteria or the first two major criteria plus a minor one [1]. These criteria are listed in Table II. A sample of bone marrow trephine biopsy (included in the WHO criteria as a major criterion) is characterized by the proliferation of all three cell lines – erythroid, granulocytic and megakaryocytic (called pancytopenia), and the presence of atypical megakaryocytes of various sizes with hyper-lobulated nuclei [1].

The diagnostic criteria proposed by the ICC are the same, except for the omission of a bone marrow biopsy in patients with sufficiently high red blood cell parameters, i.e. Hgb > 18.5 g/dL or Hct $> 55.5\%$ (men) and Hgb > 16.5 g/dL or Hct $> 49.5\%$ (women), the presence of *JAK2* mutation and decreased Epo levels [2]. However, it should be emphasized that only histopathological examination of the bone marrow is able to detect the presence of fibrosis (present in c.20% of PV patients), characteristic of those patients at higher risk of transformation to myelofibrosis [1, 16].

A cytogenetic assessment of bone marrow does not make up part of the diagnostic standard for PV. At the time of diagnosis, uncharacteristic aberrations are found in c. 15% of patients [21]. The most common include trisomy 8, trisomy 9, del (13q), and del (20q). The frequency of karyotype abnormalities increases over time, reaching

Table II. Diagnostic criteria of polycythemia vera according to World Health Organization 2022 [1]

Criteria	
Major	1. Hgb >16.5 g/dL (men); >16.0 g/dL (women) or symptoms indicative of increased red blood cell volume or Hct >49% in men and >48% in women or an increased red blood cell mass (>25%) 2. Hypercellular bone marrow with signs of erythroid, granulocytic, and megakaryocytic trilineage proliferation 3. V617F <i>JAK2</i> mutation or <i>JAK2</i> mutation in exon 12
Minor	1. Serum Epo concentration below normal

Hgb — hemoglobin; Hct — hematocrit; Epo — erythropoietin. For the diagnosis of PV, it is necessary to meet all three major criteria or major criteria 1) and 2) plus the minor one

80% in patients with a disease existing for more than 10 years [16].

Testing for the detection of *JAK2* mutations makes up a very important element of the diagnosis. In 96% of patients with PV, a *JAK2* gene V617F mutation is found in exon 14. In patients who do not have a V617F mutation, testing for mutations in exon 12 of the *JAK2* gene kinase should be performed [7, 9]. Non-canonical mutations are detected in a very small proportion of patients [10]. Testing for these mutations is not routinely performed, and neither is the determination of the variant allele frequency (VAF) in the *JAK2* gene. In the era of drugs which modify the natural course of the disease and achieve molecular remission (MR), the measurement of VAF is recommended at the diagnosis or at the start of treatment with interferon α (IFN α) or ruxolitinib (RUX) in order to trace the kinetics of changes during therapy [23]. To date, studies of the kinetics of VAF alterations of the *JAK2* gene have been conducted as part of ongoing clinical trials, and there are no clear recommendations about how often they should be performed in routine clinical practice [23]. In patients with PV, NGS testing to detect the presence of additional mutations is not routinely performed. However, it is known that mutations such as *ASXL1*, *SRSF2*, *IDH1/2*, *RUNX1* are present in c.15% of patients, something which is associated with an unfavorable prognosis and increases the risk of transformation to AML and PPV-MF [20, 23]. It is advisable to store the DNA or RNA material for future evaluation, especially in younger patients. NGS testing may also be considered in patients with a clinical picture indicative of PV and with the absence of *JAK2* mutations.

Patients with a history of hemorrhagic diathesis should be diagnosed for acquired von Willebrand syndrome (avWS) with a qualitative assessment of von Willebrand factor (vWF) — vWF:RCo or vWF:GPIb [23].

Evaluation of treatment response

Currently, the aim of PV treatment is not only to improve altered peripheral blood parameters (Hgb, Hct, leukocyte and platelet counts), but also to reduce the severity of subjective symptoms associated with disease activity.

Depending on the type of therapy undertaken, the methods of assessing the results are diverse, comprising:

- Reduction in Hct and Hgb concentration;
- Restoration of normal leukocyte and platelet counts;
- Reduction in severity of complaints based on MPN-SAF TSS (MPN Symptom — Assessment Form Total Symptom Score) scores;
- Reduction of spleen size;
- Facultative assessment of molecular response during therapy.

Table III sets out the response criteria proposed by European LeukemiaNet and IWG-MRT in 2013 [38, 39]. It should be emphasized that evaluation of samples of bone marrow trephine biopsy is only justified in clinical trials. In practice, this is only performed when there are clinical or laboratory signs suggestive of disease progression.

The assessment of molecular response is not yet used in clinical practice and it does not form the basis for therapeutic decision-making. For the purposes of clinical trials, it has been assumed that a complete molecular response (CMR) should be recognized in the case of complete eradication of the aberration demonstrated previously, whilst a partial molecular response (PMR) should be recognized in the case of a $\geq 50\%$ reduction in VAF. However, PMR can only be diagnosed in patients with baseline VAF $\geq 20\%$.

When HU therapy is used, c.20% of patients develop intolerance or resistance to the medication. The proposed criteria for the diagnosis of this condition are listed in Table IV [31].

Results of studies evaluating efficacy of phlebotomy for treatment of PV

Phlebotomy, makes up a significant part of PV treatment on account of the fast reduction of the Hct value, alleviating the disease symptoms, and also because of its lack of cytotoxic potential [23, 40]. The results of this procedure comprise a secondary absolute iron deficiency condition, whereas the primary goal is the reduction of Hct to <45% in men and <42% in women [41, 42]. According to the CY-TO-PV study, maintaining Hct below 45% with phlebotomy or HU significantly reduces the risk of cardiovascular death and major thromboembolic complications [25]. Current

Table III. Revised evaluation criteria of treatment response in patients with polycythemia vera proposed by European LeukemiaNet and IWG-MRT in 2013 [38, 39]

Response evaluation criteria	
A	Resolution of disease-related signs for ≥ 12 weeks, including hepatosplenomegaly in physical examination, significant improvement of large disease-related symptoms ^a
B	Improvement of peripheral blood parameters for ≥ 12 weeks, including Hct $< 45\%$ without need for bloodletting, PLT ≤ 400 G/L WBC < 10 G/L, absence of leukoerythroblastosis in blood
C	Absence of signs of progressive disease or new hemorrhagic or thrombotic complications
D	Bone marrow histological remission including disappearance of megakaryocyte hyperplasia and absence of $> \text{grade } 1$ reticulin fibrosis
Response evaluation	
Complete remission	All criteria are met
Partial remission	A + B + C criteria are met
No remission	Any response which does not meet criteria of partial remission
Progressive disease	Transformation to PPV-MF, MDS or AML ^b

AML – acute myeloid leukemia; Hct – hematocrit; IWG-MRT – International Working Group - Myeloproliferative Neoplasms Research and Treatment; MDS – myelodysplastic syndrome; MPN-SAF TSS – MPN Symptom Assessment Form Total Symptom Score; PLT – platelets; PPV-MF – post polycythemia vera myelofibrosis; WBC – leukocyte count; WHO – World Health Organization

Remarks: ^a Evaluation of disease severity should be performed with use of MPN-SAF TSS. A diagnosis of sustained remission/disease remission is possible in cases of a response persisting at least 12 weeks. Significant improvement in symptom severity is defined as a reduction in disease-related symptom severity ≥ 10 points assessed with use of MPN-SAF TSS.

^b Diagnosis of progression to PPV-MF: diagnosis of a specific form of myelodysplastic syndrome or acute myeloid leukemia should be confirmed on basis of criteria proposed by IWG-MRT and WHO 2016.

Table IV. Hydroxyurea intolerance and resistance criteria [31]

Resistance	Intolerance
After 3 months of treatment with a dose of ≥ 2 g/d (or 2.5 g/d in persons > 85 kg body mass): need for phlebotomy in order to maintain Hct $< 45\%$ OR PLT > 400 G/L AND: WBC > 10 G/L OR Less than 50% reduction in the size of the spleen palpable under left costal margin in palpation examination (refers to initial spleen size ≥ 10 cm below left costal margin) OR lack of resolution of any symptoms related to splenomegaly OR ELN criteria* After 3 months of treatment with any HU dose: PLT $> 1,000$ G/L OR microcirculation symptoms OR increasing leukocytosis ($\geq 100\%$ increase if baseline WBC < 10 G/L OR $\geq 50\%$ increase if baseline WBC > 10 G/L OR persistent leukocytosis > 15 G/L After one year of HU treatment in a tolerated dose: symptomatic or increasing splenomegaly (by > 5 cm under left costal margin (palpation assessment) OR need for ≥ 6 phlebotomies to maintain Hct $< 45\%$ OR increased constitutional symptoms (HU dose ≥ 1.5 g/d for ≥ 4 months): MPN-SAF TSS ≥ 20 points OR untreatable persistent skin itching for at least 6 months	During treatment with lowest HU dose making it possible to achieve at least a partial clinical response*: ANC $< 1,000$ G/L OR PLT < 100 G/L OR Hgb < 10 g/dL During treatment with any HU dose: Development of one of following symptoms: lower leg ulceration OR nonmelanoma skin carcinoma OR skin and mucosal symptoms vascular complications: clinically significant bleeding, venous or arterial thrombosis gastrointestinal symptoms pneumonia or fever each HU intolerance other than grade 3 or 4 or prolonged grade 2 HU toxicity according to CTCAE

*Clinical response criteria in polycythemia vera according to ELN. ANC – absolute neutrophil count; CTCAE – Common Terminology Criteria for Adverse Events; ELN – European LeukemiaNet; Hct – hematocrit; Hgb – hemoglobin; HU – hydroxyurea; MPN-SAF TSS – Myeloproliferative Neoplasm Symptom Assessment Form Total Symptom Score; PLT – blood platelets; WBC – leukocytes

recommendations indicate the use of phlebotomy together with ASA especially in the treatment of low-risk PV and in younger patients. In recent years, the role of phlebotomy has been increasingly debated, mainly due to the increased risk of embolic/thrombotic complications compared to other therapies, the suboptimal efficacy in maintaining a stable Hct <45%, and the impact on quality of life.

A retrospective study of a population of 533 patients with PV treated with HU showed that intensive phlebotomy, defined as more than three uptakes per year, was associated with an increased risk of thrombosis [37]. MAJIC PV is another study, suggesting that the average number of flebotomies within the first three years of therapy correlates with a shorter time to first thrombotic incident [43]. The results of this study also suggest that new drugs, such as JAK inhibitors or ropeginterferon alpha 2b (ropegIFN α -2b), may offer a valuable alternative to conventional treatments (including phlebotomy), offering similar efficacy in controlling Hct and potentially reducing the risk of thrombotic complications [43, 44]. Inadequate control of Hct levels and hyperplasia secondary to iron deficiency may turn out to be risk factors for embolic incidents in this patient population [45]. A further problem is the lack of data confirming that phlebotomy can independently maintain stable Hct levels in low-risk PV [46]. Additionally, the PVSG and ECLAP studies have shown that HU cytoreductive therapy is more effective in protecting against thrombosis than phlebotomy alone [47]. It is noteworthy that phlebotomies do not control leukocytosis, which is associated with an increased risk of arterial thrombosis, nor have the potential to reduce VAF *JAK2V617F* and, therefore, they cannot modify the course of the underlying disease, including the rate of thrombotic complications, whilst their risk increases with VAF values >50% [46].

Iron deficiency, which is secondary to phlebotomy, is connected with an excessive production of HIF2- α (hypoxia-inducible factor 2 α), which stimulates the self-reproduction of hematopoietic stem cells (HSC) and increases the release of CD34+ cells from the bone marrow. These cells, when deposited in the spleen and other tissues, increase extramedullary hematopoiesis [48]. Megakaryocytes taken from iron-deficient individuals show increased expression of the HIF2- α protein compared to cells taken from non-iron-deficient individuals [49]. HIF2- α stimulates the expression of vascular endothelial growth factor (VEGF), which enhances megakaryocytopoiesis [49].

Improvement of the patients' quality of life constitutes a vital objective of PV therapy [46, 50]. Phlebotomy, by means of reducing the Hct values, may reduce symptoms associated with hyperviscosity, but it cannot reduce the severity of pruritus or decrease splenomegaly. Chronic iron deficiency induced by phlebotomies can cause headaches, insomnia, difficulty in concentrating, dizziness, and restless legs syndrome [51, 52]. Additionally, in children and

pregnant women, there is a risk of growth impairment, and there is a risk of delay of intellectual development in children and foetuses. Phlebotomies are commonly used in children, but the target Hct values are not clearly defined; the most commonly recommended Hct values for children are reductions of less than 45% and 48% [53]. In pregnancy, the target for phlebotomy is an Hct value <45% [54].

In conclusion, phlebotomy remains an important tool in the treatment of PV, but its use should be considered and tailored to the individual patient.

Results of studies evaluating efficacy of hydroxyurea

So far, HU has been the standard first line cytoreductive treatment used in order to decrease blood cell numbers in high risk PV patients [55]. HU therapy effectively achieves rapid cytoreduction in this patient group and has a favorable safety profile.

About 90% of patients respond to HU therapy, with 24% achieving a complete response and 66% a partial response, but 25% of the treated group develop resistance or intolerance, so some patients still require periodic phlebotomies to maintain the desired Hct level [47, 56]. Around 25% of patients discontinue therapy, most commonly due to a lack of response or a suboptimal response. Other reasons for discontinuation of HU therapy include drug intolerance or disease progression [57–61]. The factors associated with HU resistance or intolerance comprise low initial Hgb levels, age over 60, and splenomegaly [60]. HU resistance and intolerance are unfavorable prognostic factors in PV, as they increase the risk of disease progression to PPV-MF or AML, although there is no evidence of direct association between HU resistance or intolerance and PV progression to AML [57, 61]. The risk of PV transformation to myelofibrosis 5 and 10 years after the onset of the disease is 3% and 17%, respectively, in patients with HU resistance or intolerance, and 1.5% and 6.7% in other patient groups. Higher rates of transformation after five years are observed in patients who fail to reduce massive splenomegaly (14% vs. 1.6%) and in those who develop cytopenias (10% vs. 1.6%) [61]. Analysis of individual resistance and intolerance criteria has shown that the development of cytopenias during HU treatment correlates with an increased risk of progression to AML (28% vs. 0.8% over five years) [61].

The PSVG study consisted of a comparison of the effect of HU and phlebotomy on the risk of thrombosis [62]. The study compared 51 patients treated with HU in combination with phlebotomy to a group of 134 patients who received phlebotomy only. The analysis made after 378 weeks of therapy showed that the incidence of thrombotic events in patients treated with HU together with phlebotomy (9.8%) was significantly lower than in the control group (32.8%, $p = 0.009$), while the incidence of AML was higher in this

group (5.9% vs. 1%, $p = 0.18$), although the difference was not statistically significant. The authors of this study suggested that younger patients who require complementary cytoreductive therapy in addition to phlebotomy should be treated with IFN α due to its lack of leukemogenic and teratogenic effects, and the possibility of achieving cytogenetic remission in some of them [62]. The efficacy of HU compared to phlebotomy was also the subject of the large, multicentre ECLAP trial comprising 1,042 subjects, treated with phlebotomy alone ($n = 342$) or HU ($n = 681$) during a follow-up in order to maintain Hct below 45% [56]. The rate of patients with a target Hct below 45% after 12 months was higher in the HU group compared to the patients treated with phlebotomy (52% vs. 31%, $p < 0.0001$). In high-risk patients (above 60 years or with a history of thrombosis), the rate of fatal and non-fatal cardiovascular events was significantly lower in the HU-treated group (4.8 vs. 8.7/100 patient-years in the HU vs. phlebotomy group), and so was the rate of transformation to myelofibrosis (0.1 vs. 1.5/100 patient-years in the HU vs phlebotomy group) and mortality (0.1 vs. 0.5/100 patient-years in the HU vs. phlebotomy group). The percentage of mortality and cardiovascular events was significantly higher in patients in the phlebotomy group who did not reach the target Hct level of <45% ($p = 0.000$) compared to the HU-treated patients [56]. Another retrospective, single-centre study involving 470 patients compared the effect of IFN α , HU and phlebotomy alone on myelofibrosis-free survival (MFS) and OS [63]. The mean follow-up period was 10 years (range 0 to 45). Out of 437 patients, 208 belonged to a high-risk group (44%). HU was administered in 189 patients (40%), IFN α in 93 patients (20%), phlebotomy in 133 patients (28%), and other cytoreductive drugs in 55 patients (12%). In low-risk patients, 20-year MFS was 84% for IFN α , 65% for HU, and 55% for phlebotomy, respectively ($p < 0.001$), and 20-year OS was 100%, 85%, and 80%, respectively ($p = 0.44$). In the high-risk group, 20-year MFS was 89% for IFN α , 41% for HU, and 36% for phlebotomy ($p = 0.19$), while 20-year OS was 66%, 40%, and 14%, respectively ($p = 0.016$). Multivariate analysis revealed that a longer duration of IFN α use was associated with a lower risk of developing myelofibrosis (relative risk [HR]: 0.91, $p = 0.012$) and a lower mortality (HR: 0.94, $p = 0.012$) [63]. The majority of study results confirm the absence of a leukemogenic effect of HU, but the long-term use of HU is associated with a 20% risk of developing skin cancers other than melanoma [64, 65].

Results of studies evaluating efficacy of interferon alpha

Interferons (IFNs) are cytokines with anti-inflammatory, immunomodulatory and anti-tumour effects. Several subtypes of these molecules function in the healthy human body, but, in this group only IFN α has a therapeutic use in

the treatment of myeloproliferative neoplasms [66]. It is characterized by a strong proapoptotic effect, also shown against progenitor cells with the presence of JAK2 kinase mutations. The forms of recombinant IFN α originally used in MPN therapy were characterized by a short half-life and an unfavourable safety profile, which was the cause of numerous treatment failures [66]. The modification of recombinant interferon by attaching a biocompatible polymer, polyethylene glycol (Peg), to the IFN α molecule allowed the half-life to be extended, thus reducing the frequency of use (to once weekly) and the toxicity [66].

A further structural modification (ropegylation) resulted in an increase in the half-life of the drug and an increase in the intervals between IFN α administrations to two weeks and a further reduction in toxicity. Peginterferons, while retaining their proapoptotic efficacy, activate the immune system to a lesser extent, leading to a significantly more favourable profile of side effects. The efficacy of modified forms of IFN α in PV therapy has been confirmed in numerous studies. Early phase II and III studies evaluating the safety and efficacy of PegIFN α 2a and 2b included groups with different MPNs, and different criteria were used to assess response. The studies analysing PegIFN α 2a included both patients with newly diagnosed high-risk PV (MPD-RC-112 study) and patients with HU resistance or intolerance (MPD-RC-111 study) [67, 68]. In the population of previously treated patients, the response rate (complete and partial remissions) achieved after 12 months of PegIFN α treatment was 60%. The medication used in the first-line treatment made it possible to achieve a 78% response rate, and this percentage was comparable to that obtained in the hydroxyurea arm. Complete hematological remission (CR) was achieved in 22% and 30% of those treated with HU and those not receiving any treatment prior to PegIFN α , respectively. In subsequent years of follow-up, the percentage of complete remissions in the IFN α -treated patients increased as opposed to the HU-treated group [67, 68].

In recent years, the results of studies conducted exclusively with ropegIFN α -2b have been published. In the PEGINVERA trial, a reduction in toxicity and an improvement in treatment efficacy were reported [69]. Most side effects were mild and transient, while severe symptoms were reported in 19.6% of patients (two depressive episodes, thyroid dysfunction, atrial fibrillation, arthritis, flu-like syndrome, febrile states, and increased transaminase activity). The percentage of CR achieved in the study was 64.3% at 2-year follow-up, while partial hematological remission (PR) was 33%. The time to CR was relatively long at 34 weeks; and the time interval to hematological response was 10 weeks. Once a response was achieved, the drug was administered at a reduced frequency *i.e.* every four weeks, with no loss of therapeutic effect. Therapy with ropegIFN α -2b resulted in a slow reduction in the mutant allele burden, and a CMR of 28.6% was achieved. The median time to CR was 82 weeks [69].

Subsequent studies (PROUD-PV and CONTINUATION-PV) comparing ropegIFN α -2b to HU confirmed the efficacy and good tolerability of this form of the drug [44, 69]. The study included 257 patients. RpegIFN α -2b was administered subcutaneously every two weeks at an initial dose of 100 μ g. After 12 months, the primary endpoint, *i.e.* CR and normalization of spleen size, was achieved in 21% of patients in the ropegIFN α -2b group and in 28% of the HU-treated patients ($p = 0.23$). CR without normalization of spleen size was observed in 43% vs. 48%, respectively ($p = 0.63$). The response rate to ropegIFN α -2b increased over time. The assessment made at 36 months revealed CR in 71% of patients treated with ropegIFN α -2b and in 51% of those receiving HU ($p = 0.012$). Moreover, CR, reduction in splenomegaly and decrease of the symptoms related to microcirculatory disorders, pruritus and headaches were observed in 53% of patients treated with ropegIFN α -2b and 38% of patients in the HU group ($p = 0.044$). A progressive reduction in VAF JAK2V617F was also observed in the ropegIFN α -2b-treated group. After 36 months of ropegIFN α -2b therapy, VAF decreased from 42.8% to 19.7%, whereas in HU-treated patients the reduction was only transitory and, at 36 months, VAF was not significantly different from the baseline values. Tolerability of ropegIFN α -2b was good. The most common grade 3 and 4 adverse events in the ropegIFN α -2b-treated group were the elevations of GGTP (gamma-glutamyltranspeptidase) (6%) and ALT (alanine transaminase) (3%), and in the HU group were leukopenia (5%) and thrombocytopenia (4%). Grade 3 and 4 depression was observed in 2% of patients in each group [44].

The efficacy of ropegIFN α -2b was also evaluated in low-risk PV patients, in whom the drug was compared to flebotomy [70]. The primary objective of the study was to assess the proportion of patients with Hct <45% and without adverse events (thrombotic complications, bleeding, progressive leukocytosis, symptomatic or very high thrombocytosis, symptomatic splenomegaly). The primary objective was achieved in 84% of patients in the ropegIFN α -2b group compared to 60% in the standard arm ($p = 0.008$). What's more, the patients in the ropegIFN α -2b group required fewer phlebotomies: 43% vs. 57%, had fewer general symptoms assessed according to the MPN Symptom Assessment Form Total Symptom Score (MPN-SAF TSS) ($p = 0.033$), and had significant reductions in splenomegaly, leukocytosis and platelet count. No differences were observed in the frequency of >3 grade adverse events in the two groups: 6% (ropegIFN α -2b) vs. 8% (phlebotomy) [70].

Results of studies evaluating efficacy of ruxolitinib

The drug registered for the treatment of PV patients manifesting resistance or intolerance to HU is ruxolitinib (RUX). In 2014, Vannucchi et al. [71] published the results of

a randomized phase III trial (RESPONSE trial) comparing the efficacy of RUX and best available therapy (BAT) in a group of 222 PV patients with splenomegaly, resistant or intolerant to HU. The study showed significantly better efficacy of RUX compared to BAT with respect to both Hct control and reduction of overall symptoms. The primary study objective of Hct control and spleen size reduction of at least 35% was achieved in 21% of patients in the RUX group, but in only 1% in the BAT group ($p < 0.001$). Hct control was observed in 60% of patients receiving RUX and 20% in the BAT group. CR was achieved by 24% of patients treated with RUX compared to 9% in the BAT group ($p = 0.003$). Better control of general symptoms was also observed among RUX-treated patients. At week 32 of the study, at least a 50% reduction in MPN-SAF TSS symptom severity was achieved by 49% vs. 5% of patients, respectively. A gradual reduction in VAF JAK2 was also observed in the RUX group (maximum mean reduction: -34.7% at week 112). The assessment of treatment complications concerned the first 32 weeks of the study, as after this time most patients were switched to the RUX arm. Treatment tolerability was good, yet herpes zoster infections were more frequently observed in patients treated with RUX (6% vs. 0%). On the basis of the results of the RESPONSE trial, RUX was registered for the treatment of PV patients resistant or intolerant to HU [71]. Similar results were observed in the RESPONSE 2 trial, which included patients with PV refractory or intolerant to HU, but without splenomegaly [72]. Hct control was achieved in 62% of patients in the RUX group and 19% in the BAT group ($p < 0.001$) [72]. However, it should be noted that in both RESPONSE studies, about one half of the patients in the BAT group received HU [71,72].

The RELIEF study assessed the efficacy of RUX compared to HU in patients who had good Hct control yet with general symptoms [73]. The primary objective of the study was to assess the proportion of patients who experienced at least a 50% reduction in MPN-SAF TSS-assessed symptoms after 16 weeks of treatment. This percentage was higher in the RUX-treated group compared to HU-treated patients (43.4% vs. 29.6%, respectively), yet the difference was not statistically significant, although RUX was significantly more effective in reducing pruritus [73].

The phase II MAJIC-PV study evaluated the efficacy of RUX versus BAT in 180 HU-resistant/intolerant patients [43]. The BAT group was treated with HU (32%), IFN α (15%), and HU together with IFN α (12%). Patients treated with RUX were significantly more likely to achieve CR (43% vs. 26%; $p = 0.02$), and their response lasted longer ($p < 0.001$). Event-free survival (EFS), defined as time free from major thrombotic/hemorrhagic episodes and transformation or death, was significantly longer in the patients who achieved CR ($p = 0.01$) and those treated with RUX ($p = 0.03$). Patients treated with RUX were more likely to have a >50% reduction in VAF JAK2 (56% of patients after

48 months of follow-up in the RUX group versus 26% of patients after a median follow-up of 36 months in the BAT group, $p < 0.001$). Infections, mostly respiratory and genitourinary infections and *herpes zoster*, were significantly more frequent in the RUX-treated patients (27 vs. 12 grade 3/4 infections, respectively). Skin squamous cell carcinoma was also more common in the RUX group (11 vs. 0, respectively) [43].

Recommendations concerning treatment of patients with PV

Irrespective of the risk group, the treatment goals for patients with PV are unchanged and include [31]:

- 1) Reduction of risk of thromboembolic complications.
- 2) Reduction of symptoms resulting from disease.
- 3) Reduction of risk of disease progression to MF and AML.

In each risk group and at each stage of treatment, efforts should be made to eliminate or minimize cardiovascular risk factors (CVRFs) independent of PV, such as the normalization of body weight, adequate control of blood pressure values, control of lipid metabolism, normalization of serum glucose levels and stopping smoking [74]. The use of hormonal contraceptives is contraindicated, whilst hormone replacement therapy should only be used when the benefits of such treatment outweigh the risk of embolic complications.

In patients with newly diagnosed PV, irrespective of risk group, phlebotomy should be performed to reduce quickly the Hct values. The frequency of phlebotomies should be adjusted to the resolution of subjective symptoms associated with polycythemia or the rate of Hct decline and the planned time period to achieve the desired Hct value, as well as the tolerability of the procedures. After each procedure, the lost blood volume should be replenished with the administration of 0.9% NaCl solution. The frequency of the procedure (every two days or twice a week) as well as the volume of successive phlebotomies (250–400 mL) should be determined individually for each patient depending on comorbidities (especially cardiovascular conditions) and the dynamics of Hct decrease after subsequent procedures [70].

Recommendations concerning treatment of low-risk PV patients

Low-risk patients are a group that requires special care when choosing a therapeutic strategy. According to classical prognostic criteria, they are young patients (<60 years old) who have never had a thromboembolic incident. In this group of patients, in addition to the reduction of the risk of thromboembolic complications and the reduction of symptoms resulting from the disease, the expected long-term course of the disease and, consequently,

the significant probability of developing both PPV-MF and AML in the future, should be taken into account.

The CYTO-PV study showed a clear benefit of the strategy of maintaining hematocrit <45%, reducing the rate of thromboembolic complications [25]. In order to reduce red blood cell parameters in patients with low-risk PV, it is advisable to perform phlebotomy first. However, it should be kept in mind that not all patients tolerate phlebotomies well, and a repetition of this procedure may lead to the development of tissue iron deficiency and secondary thrombocytosis resulting in increased microvascular symptoms [75]. The most recent European LeukemiaNet (ELN) recommendations have identified groups of patients with low-risk PV who may benefit from the inclusion of cytoreductive treatment (see Tab. V) [31]. The choice of treatment in this group of patients should take into account the expected long-term exposure to the selected drug. Due to the time-dependent skin complications, as well as the lack of effect of HU on the eradication of the *JAK2* mutant clone, the use of this drug is not recommended in patients with low-risk PV. Considering the results of the Low-PV study, which confirmed the safety of ropegIFN α -2b and proved its superiority in hematocrit control over phlebotomy treatment, ropegIFN α -2b is the drug of choice in this patient group [46].

Recommendations concerning treatment of high-risk PV patients

High-risk PV patients require cytoreductive treatment in addition to phlebotomies [31]. The drugs of first choice comprise HU or IFN α . The initial dose of HU is usually 0.5 g twice daily, and then it should be modified to keep Hct below 45%. Approximately 25% of patients experience drug ineffectiveness or intolerance. Adverse effects that HU can cause include cytopenias, mucosal and skin ulcerations, gastrointestinal intolerance, fever, and skin lesions including non-melanoma skin tumors. It is important to emphasize the need to carefully collect medical history and assess the skin condition during and assess the skin condition during follow-up visits of patients treated with HU.

Currently, the drug recommended for first-line PV therapy, especially in younger patients, is IFN α [23]. It is believed that its use may lead to bone marrow histology response, a reduction in VAF *JAK2*, and a decrease in the incidence of PV transformation to MF [23]. In 2020, the European Medicine Agency (EMA) approved ropegIFN α -2b for the treatment of patients with PV without significant splenomegaly.

In Poland, ropegIFN α -2b therapy is reimbursed for the following indications: 1) young high-risk patients with indications for cytoreductive therapy; 2) patients intolerant of or refractory to HU therapy; and 3) pregnant women.

It should be noted that the ELN experts claim that patients who are classified as low risk according to the

Table V. Recommendations concerning cytoreductive pharmacotherapy in low-risk PV patients proposed by ELN experts [31]

	Cytoreductive treatment in low-risk PV patients
Recommended	1. Poor tolerance to phlebotomy, defined as recurrent syncope episodes following phlebotomy in spite of preventive measures, or hematophobia, leading to avoidance behaviours or significant difficulties in obtaining venous access (consensus: 100%) 2. Progressive symptomatic splenomegaly (spleen increase by >5 cm within last year) provided that transformation to myelofibrosis has been ruled out (consensus: 85%) 3. Persistent leukocytosis (leukocyte count >20 G/L confirmed at 3 months without therapy (consensus: 85%)
To be considered	1. Progressive leukocytosis (increase by at least 100%, with baseline count <10 G/L, or increase by at least 50%, with baseline count >10 G/L) and persistent leukocytosis >15 G/L, confirmed within 3 months (consensus: 80%) 2. Significant thrombocytosis (>1,500 G/L), bleeding manifestations regardless of platelet count, or both (consensus: 85%) 3. Suboptimal hematocrit control following phlebotomy, i.e. need to perform at least 6 phlebotomies per year for at least 2 years in maintenance therapy after obtaining hematocrit below 45% in induction therapy (consensus: 80%)
May be considered	1. Severe general symptoms (total symptoms score in MPN-SAF TSS form ≥ 20 points) or persistent and severe itching (itching score ≥ 5 points), which do not resolve after phlebotomy, antiplatelet therapy or antihistamine medications (consensus: 93%) 2. Individualized in cases of patients with significant cardiovascular risk provided that primary prophylaxis was applied (consensus: 85%)

ELN – European LeukemiaNet; MPN-SAF TSS – Myeloproliferative Neoplasm Symptom Assessment Form Total Symptom Score; PV – polycythemia vera

conventional index, but who are burdened with the factors listed in Table V, should be considered as high-risk patients (requiring the implementation of cytoreductive treatment) [23]. The preferred drug in this group of patients is ropegIFN α -2b. When considering whether to administer ropegIFN α -2b, it is necessary to be aware of potential contraindications to this drug such as end-stage renal failure (glomerular filtration rate [GFR] <15 mL/min/1.73m²), severe cardiovascular disease (uncontrolled hypertension, congestive heart failure in NYHA [New York Heart Association] class ≥ 2 , severe cardiac arrhythmias, significant coronary stenosis, unstable angina pectoris), the presence of autoimmune disease, and serious psychiatric disorders, especially severe depression with a history of suicidal ideation or attempted suicide. Compensated cirrhosis (Child-Pugh stage A) is not a contraindication for ropegIFN α -2b [76]. During the treatment, regular mood assessment and control of biochemical parameters are required: the levels of aspartate aminotransferase (AST), ALT, GGTP, thyrotropic hormone (TSH), and creatinine. The dose is adjusted individually for each patient starting at the recommended dose of 100 μ g (or 50 μ g in patients receiving other cytoreductive treatment). The dose should be gradually increased by 50 μ g every fortnight (with a concomitant, gradual reduction of other cytoreductive treatment, if applicable) until stabilization of hematological parameters is achieved. The maximum recommended single dose is 500 μ g injected every fortnight [76]. In a clinical trial conducted on polycythemia

vera, the mean adjustment period for an individual dose of ropegIFN α -2b was 3.7 months of drug administration and for HU was c.2.6 months [76].

In patients with resistance or intolerance to HU (the criteria are listed in Table IV), treatment with a previously unused drug is recommended: RUX or IFN α [23]. When RUX is administered at an initial dose of 10 mg bid, a higher propensity for infectious complications (mainly *herpes zoster*) and skin lesions (non-melanoma skin cancers) must be taken into consideration [43].

In older persons, i.e. >65 years, or patients refractory/ /intolerant to other therapies, busulfan can be used [23]. However, the use of this drug requires special caution due to its potential irreversible myelotoxic effect. Treatment is initiated at a dose of 2–4 mg/day and should be reduced if the platelet count falls below 150 G/L or the leukocyte count below 5 G/L, while a reduction in platelet count below 100 G/L or leukocyte count below 3 G/L requires discontinuation of the drug. The effects of busulfan may persist for a long period after discontinuation of therapy.

Therapy recommendations for patients with PV are listed in Figure 1.

Antithrombotic prophylaxis and treatment

The most important preventive measure is to maintain Hct values <45% in men and <42% in women. Recent expert recommendations recommend even lower target Hct values

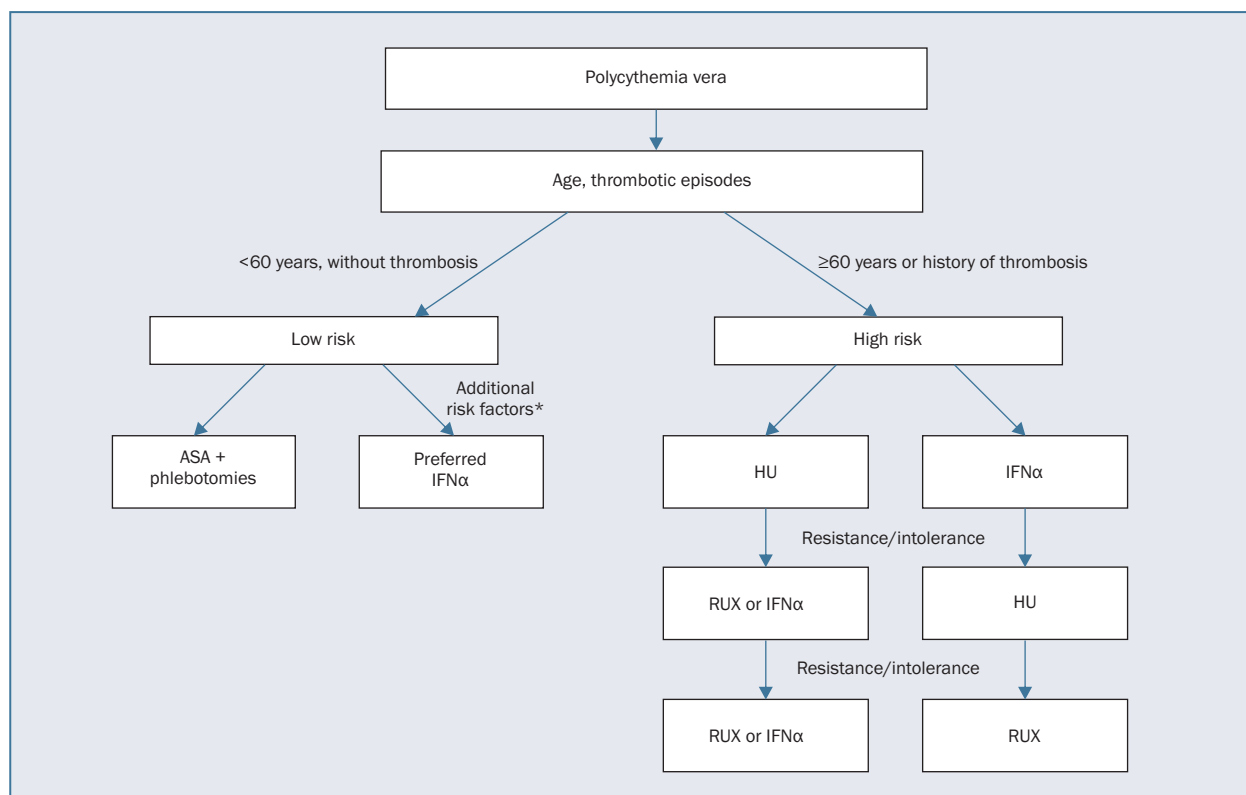


Figure 1. Recommendations for the treatment of polycythemia vera. *As shown in Table V. ASA – acetylsalicylic acid; IFNα – interferon alpha; HU – hydroxyurea; RUX – ruxolitinib

(40–42%) for patients with persistent symptoms of blood hyperviscosity, such as erythromelalgia, transient ocular ischemic episodes, headache, dizziness or amaurosis fugax, despite lowering Hct <45%, if there is a demonstrable benefit of such an option [77].

In low-risk PV patients, the administration of low-dose ASA (40–100 mg) once per day has a documented anti-coagulant efficacy [78, 79]. Antiplatelet treatment reduces the incidence of venous thrombosis in patients with a *JAK2* gene mutation present, as well as the incidence of arterial thrombotic episodes in patients with coexisting cardiovascular risk factors [78]. The administration of ASA twice per day should be considered in patients with co-occurring cardiovascular risk factors, leukocytosis or the presence of microvascular symptoms, despite low doses of the drug. ASA should be administered twice per day in high-risk patients after an episode of arterial thrombosis [80]. Cardiovascular risk should be assessed at baseline and once per year with a validated risk assessment scale, e.g. QRISK score or SCORE risk [74, 81]. The details of thromboprophylaxis are presented in Table V.

Treatment of acute thrombotic episodes

The therapy of acute thrombotic complications should follow current guidelines on thrombosis treatment. In cases

where the thrombotic complication is the first manifestation of the disease, cytoreductive treatment should be implemented immediately. The management should also include phlebotomy to reduce Hct to <45% [80]. In patients with newly diagnosed PV, as well as in those who develop thrombotic complications during cytoreductive treatment, the therapeutic management should be optimized so that the blood morphotic values remain within the desired range. Thus, in cases of arterial thrombosis, treatment with low doses of ASA should be implemented immediately, together with specialist consultations (e.g. a cardiologist in a case of coronary thrombosis, a neurologist/neurosurgeon in a case of cerebral arterial thrombosis) – it is necessary to follow the current specialist recommendations [81, 82]. In cases of venous thrombosis, it is advisable to start anti-coagulation treatment with low-molecular-weight heparins, with a switch to vitamin K antagonist (VKA) treatment according to current guidelines [81, 82].

In patients with an unprovoked episode of venous thrombosis, it is recommended to use long-term anticoagulation with VKA or DOACs (direct oral anticoagulants) for secondary prevention, taking into account the current hemorrhagic risk, including avWS [80]. A similar approach should be applied in cases where visceral venous thrombosis is confirmed and cerebral venous thrombosis is diagnosed [80].

Table VI. Antithrombotic prophylaxis in patients with PV

All patients	
1. Phlebotomies until values adequate to maintain Hct <45% in men and <42% in women are achieved	
2. ASA once per day	
Low-risk patients (<60 years, no history of thrombosis)	High-risk patients (history of thrombosis or age >60)
ASA twice per day should be considered in following cases: a) microvascular symptoms despite administration of this drug once per day; b) co-existence of cardiovascular risk factors; c) leukocytosis	1. Cyto-reductive therapy 2. In patients with a history of arterial thrombosis, ASA should be administered twice per day and anticoagulation treatment should be initiated

ASA – acetylsalicylic acid; Hct – hematocrit; PV – polycythemia vera

In recent years, DOACs have been increasingly used for thromboprophylaxis. Studies of the efficacy of DOACs carried out in a cancer population indicate that these drugs have significant efficacy and a good safety profile [83]. Unfortunately, there are no randomized trials comparing the activity of DOACs to that of VKAs in MPN patients. However, increasing data indicates similar efficacy in this patient group as well [84–86]. Weronka et al. [84] used DOACs in 48 patients with PV (70.8%) and ET who experienced venous thromboembolism (VTE). Patients were given apixaban (39.6%), rivaroxaban (33.3%) and dabigatran (27.1%). During 30 months of follow-up (range 20.5–41.5), there were four thrombotic episodes (3.3 per 100 patient-years) and one major bleeding episode [84]. Serrao et al. [85] used DOACs in 71 patients with MPN (including 15 with PV) for atrial fibrillation (AF) or VTE. At 12-month follow-up, no patient had thrombotic complications or a major hemorrhagic episode [85]. A multicentre observational study carried out by Barbui et al. [86] included 442 patients with MPN (including 178 with PV). Patients received DOACs (dabigatran, apixaban, rivaroxaban, edoxaban) for AF (n = 203) or a history of VTE (n = 239). After a median follow-up of 1.7 years, there were 10 major thrombotic episodes (2.1 per 100 patient-years) in the AF group and 22 thrombotic complications (4.5 per 100 patient-years), including 17 venous and five arterial ones, among patients with VTE. In the entire group, 26 major bleeding complications were observed (14 in AF patients and 12 in VTE patients), representing 3% and 2.3% per year, respectively. Bleedings most often occurred in the gastrointestinal tract and were mainly observed in patients with MF, more frequently in those treated with dabigatran [86]. The incidence of thrombotic and hemorrhagic complications observed in these studies is similar to that of prophylaxis with VKA, but the retrospective nature of the studies should be emphasized. The decision on the type of thromboprophylaxis should be taken considering thrombotic and hemorrhagic risk factors, renal and hepatic function status, medications taken, and patient preference.

Pregnancy in a patient with PV

Thrombotic complications make up a major cause of morbidity and mortality in patients with MPNs [87]. Since pregnancy is a state of increased thrombotic readiness, the risk of thrombotic complications in pregnant MPNs is higher than in the general population [54, 87, 88]. An analysis of 129 pregnancies in 60 patients with MPNs revealed that 68.2% of pregnancies ended in obstetric success, and the main complications concern the fetus: miscarriages (31.8%) and preterm births (17.8%) [89]. Pregnant women with MPNs are at risk of thrombotic and hemorrhagic complications; however, an analysis conducted by Landt-blom et al. [90] between 1973 and 2018 found no statistically significant difference in these complications between patients with and without MPNs.

The treatment of a pregnant woman with MPNs should be carried out in close collaboration between hematologist and obstetrician-gynecologist. The care of a pregnant woman with MPN should begin before conception: the patient (and her partner) should be informed of the risks of pregnancy, and her cardiovascular risk factors and blood count parameters should be assessed. Based on them, the cyto-reductive therapy should be modified or planned accordingly (maintenance or introduction of IFNα) [91].

The cyto-reductive drug of choice, due to its safety for the proper development of the fetus and the course of pregnancy, is IFNα [92, 93].

However, this therapy is not recommended for all pregnant women with MPN but only for those at high risk, i.e. in whose cases there were:

- 1) ≥1 miscarriage of unclear etiology <10 weeks' gestation (hbd);
- 2) ≥1 death of unclear etiology of a morphologically normal fetus ≥10 hbd;
- 3) ≥1 preterm delivery of a morphologically normal fetus <34 hbd, due to severe pre-eclampsia, eclampsia or placental insufficiency;
- 4) a history of peripartum hemorrhage requiring transfusion [94].

In addition, the classification of a patient into the high-risk group is determined by the result of a Doppler ultrasound examination of the uterine arteries at 20 hbd. An abnormal test result indicates placental insufficiency (pulsation index >1.4) due to vascular flow disturbances [80, 94–96].

HU and VKA, due to their potential teratogenic effects, should be discontinued at least three months before conception (both in the affected woman and in the partner with MPN). There is no conclusive data on the safety of anagrelide (ANA) in pregnancy, but due to the risk of thrombocytopenia in the fetus, this drug should be discontinued in the affected woman three months before attempting to conceive [80, 94, 95]. However, it should be noted that there have been reports of normal pregnancy outcomes in patients with MPNs who received HU or ANA during the first trimester of pregnancy [97].

Some generally recognized risk factors for complications in pregnant patients with MPN include a history of arterial or venous thrombosis, and also hemorrhage. A high platelet count of more than 1,000 G/L or 1,500 G/L is also considered a risk factor, primarily due to the risk of avWS. The risk of complications is also increased with high leukocytosis, Hct $>45\%$ despite phlebotomies, concomitant diseases such as diabetes, hypertension, and older maternal age (>35 years) [80, 94, 95]. At present, the status of directional mutations does not determine the therapy of pregnant women with MPN.

In all pregnant women with MPN, it is recommended to make a blood count check every four weeks until 24 hbd and then every two weeks. Testing for avWS (ristocetin activity) is advisable, especially in patients with PLT count $>1,000$ G/L. It should be borne in mind that both PLT and Hct counts tend to spontaneously decrease during pregnancy, and iron supplementation is associated with a risk of increased red cell parameters and is therefore not recommended as standard [80, 94–96].

In patients with PV, phlebotomy is additionally recommended in order to maintain Hct $<45\%$ or its normal range applicable for the trimester of pregnancy (31–41% in the first trimester, 30–38% in the second trimester, and 28–39% in the third trimester) [80]. Strict monitoring of blood pressure and regular urinalysis are necessary. If the patient is given low-molecular-weight heparin (LMWH) in a therapeutic dose, it is advisable to determine the anti-X activity every two months [94]. Fetal ultrasound is another important examination in pregnancy to assess normal growth. This assessment should be performed at 32 and 36 hbd [91].

According to the published data, proper management of a pregnant patient with PV based on low doses of ASA and LMWH or IFN α alone or in combination with ASA or LMWH, significantly increases the live birth rate from 47.6% to 78.2%. Thus, a low-dose ASA is recommended in every

pregnant woman with MPNs provided that there are no contraindications [89,98]. ASA should be administered throughout pregnancy with the exception of the last two weeks before the planned delivery, when a the switch to prophylactic dose of LMWH should be made. The last dose of heparin should be given 12 hours before delivery, another dose c.12 hours after delivery, followed by prophylactic use of LMWH and ASA for six weeks postpartum, in view of the particularly high risk of thrombotic complications characteristic of this period. Prophylaxis with LMWH at the standard prophylactic dose (e.g. enoxaparin 40 mg once a day) should be initiated as soon as possible after pregnancy is confirmed in all women with MPN and a history of venous thrombosis or obstetric failure [80]. In these patients, doubling the dose of LMWH after 16–20 hbd is recommended. In patients with a history of an episode of arterial thrombosis, UK recommendations suggest an intermediate dose of LMWH (e.g. enoxaparin 40 mg bid) [80, 99]. Therapeutic doses of LMWH are administered for thrombotic complications during pregnancy [80, 94–96].

In vitro fertilization (IVF) methods are acceptable, although there are no established standards of therapeutic management in this group of patients. Currently, LMWH is recommended from the start of hormonal therapy up to 12 hbd, with a short interval for oocyte retrieval. IVF is an identified risk factor during pregnancy [91].

In conclusion, all women of childbearing age with MPNs should be informed about possible complications in pregnancy and the need to plan for it. HU and ANA should be discontinued at least three months before pregnancy. A close collaboration between the hematologist and the obstetrician-gynecologist is essential for the correct management of a pregnancy in a patient with MPNs. During pregnancy, it is necessary to check peripheral blood count parameters, fetal ultrasound, and assessment of uterine artery flow at 20 and 24 hbd. In every patient, low-dose ASA should be introduced as soon as possible (preferably before pregnancy) as routine thromboprophylaxis and continued until the end of the postpartum period. The addition of LMWH is recommended if there are additional risk factors for thrombotic complications (thrombotic incident, a history of obstetric failures) and for six weeks postpartum in all women with MPN. Close monitoring of Hct levels $<45\%$ (or even $<42\%$) is recommended for PV. Cyto-reductive therapy is reserved for women with MPN with a high risk of thrombotic complications, and the drug of choice is IFN α . There are no clear recommendations for IFN α therapy during breastfeeding.

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Authors' contributions

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JG-T, TS, MB, MS, KL — Advisory board membership (Novartis, Orphan AOP, GSK), lectures: Novartis, AbbVie, Orphan AOP, Pfizer. AG — lectures: Novartis, AbbVie, Orphan AOP, Pfizer. PS, OCH — no declaration.

Supplementary material

Not applicable.

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Mutations of variable parts of immunoglobulin heavy chains (*IGHV*) – assay methodology and recommendations on result reporting

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Abstract

Currently, the evaluation of mutational status of variable region of immunoglobulin heavy chain gene (*IGHV*) makes up one of the key prognostic and predictive tests in patients with chronic lymphocytic leukaemia (CLL), performed before the initiation of first line therapy. This publication presents the first edition of the Polish recommendations of the Molecular Haematology Section of the Polish Society of Human Genetics (PTGC) concerning the methodology of *IGHV* mutation status determination and the principles of reporting the results based on the recognised international standards.

Keywords: chronic lymphocytic leukaemia (CLL), *IGHV* mutation, recommendations, reporting

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Introduction

Currently, the evaluation of mutational status of variable region of immunoglobulin heavy chain gene (*IGHV*) makes up one of the most important prognostic and predictive tests in patients with chronic lymphocytic leukaemia (CLL), performed before the initiation of first line therapy.

In accordance with the international recommendations of the National Comprehensive Cancer Network (NCCN) and the World Health Organisation (WHO), this test has become the standard recommended in all CLL patients and also in patients with small B lymphocytic lymphoma (SLL) before the initiation of first line therapy [1, 2]. The Molecular Haematology Section of the Polish Society of Human Genetics (PTGC) recommends this test to be performed as part of initial diagnostics in patients with a diagnosis of CLL or SLL, provided that the rate of peripheral clonal

lymphocytes is sufficient. Given the therapeutic options currently available, which depend among other things on the *IGHV* mutation status, it is essential to ensure that all patients have access to this test and that it is performed in accordance with international quality standards.

The standardisation of the methodology for determining the *IGHV* mutation status and the principles for reporting the results are important elements of CLL diagnostics, and also facilitate communication between clinicians and diagnostic specialists.

Significance of *IGHV* mutation status in CLL

Depending on the population, it is estimated that 50–55% of CLL patients have an unmutated *IGHV* status, which is associated with a poorer prognosis. The remaining patients' mutated *IGHV* status represents a group with

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a better prognosis. The *IGHV* mutation status indicates the stage of B lymphocyte differentiation at which cancer transformation occurred. If the *IGHV* gene has undergone numerous mutations (and that therefore there is mutated *IGHV* status), this means that the CLL lymphocytes have passed through the proliferation centres of the lymph follicles and that somatic hypermutation (SHM) of the *IGHV* genes has occurred, together with the processes of differentiation and maturation under the influence of antigenic stimulation. These cells are characterised by a more stable genome, a lower proliferative index, and lower expression of ZAP-70 and CD38, which have long been regarded as negative prognostic factors in CLL. In leukaemia cells with mutated *IGHV*, additional aberrations with more adverse significance are less frequent, which contributes to a better prognosis.

If the *IGHV* genes did not undergo intensive mutation processes, the cancer transformation occurred before the B lymphocytes reached the proliferation centres of the lymph follicles. The absence of *IGHV* hypermutation is associated with a poor prognosis, high expression of ZAP-70 and CD38, and aberrant apoptotic profile of cancer cells. Moreover, it is associated with the presence of additional genetic alterations that negatively affect prognosis, which result from genetic instability of these cells [3].

Indications for testing

The test should be performed in patients with a cytometrically or histopathologically confirmed diagnosis of CLL or SLL for prognostic purposes and/or targeted therapy eligibility. Furthermore, due to the temporal stability of *IGHV* SHM status, baseline determination of *IGHV* clonotype is essential for future molecular monitoring of measurable MRD (minimal residual disease) and confirmation of progression or transformation of CLL into aggressive lymphoma [4]. The Molecular Haematology Section of the Polish Society of Human Genetics does not recommend *IGHV* testing in cases of monoclonal B-cell lymphocytosis (MBL) which do not meet the criteria of CLL/SLL diagnosis.

Treatment of patients with chronic lymphocytic leukaemia

The following agents are used in the treatment of CLL:

- ibrutinib, acalabrutinib, zanubrutinib (in monotherapy or in combination),
- obinutuzumab (in combination with chlorambucil or venetoclax),
- venetoclax (alone or in combination with obinutuzumab or rituximab).

The above therapeutic options for patients with CLL are reimbursed by the National Health Fund (drug programme

B.79 [5]). Diagnostic and therapeutic management and treatment regimens are also included in the PALG-CLL expert panel guidelines [6].

Material

In the case of a confirmed diagnosis of CLL, the test material is a peripheral blood sample collected into a sterile tube containing K2-EDTA in a volume of at least 9 mL (it is important to ensure the correct ratio of blood volume collected to the anticoagulant in the tube). In the case of a diagnosis of SLL, when the absolute WBC count is less than 11,000/ μ L and the percentage of lymphocytes is less than 50%, it is recommended that other material with clonal lymphocyte infiltration is collected, such as bone marrow in a volume of 2–3 mL collected in a sterile tube containing K2-EDTA (provided lymphoproliferative features are present) or pathologically altered fresh tissue sample collected in saline or alcohol (formalin is not eligible). The choice of method *i.e.* fine-needle biopsy or resection of the involved node, is up to the clinician, who, in consultation with the molecular/genetic laboratory, decides which material would be most suitable.

Necessary documentation

The documentation accompanying the sample should contain the data required directly for *IGHV* testing. General recommendations concerning the documentation of molecular tests were specified in Annex 4 of the Regulation of Poland's Minister of Health of 23 March 2006 on quality standards for medical diagnostic and microbiological laboratories, which was in force until the end of 2023 [7]. Presently, the Ministry of Health is preparing new guidelines.

The test order form should contain the following items:

- diagnosis: CLL, SLL (in cases of MBL, the Molecular Haematology Section of PTGC does not recommend *IGHV* mutation testing);
- disease status description: patient before treatment/ /disease progression/Richter's transformation (RT)/ /MRD (solely for high-sensitivity NGS techniques);
- specification of sample source *i.e.* peripheral blood, bone marrow, biopsy sample;
- valid result of a blood test documenting the percentage and/or absolute leukocyte count in the sample, *e.g.* the last CBC, flow cytometry, blood smear or histopathological result (in cases of bone marrow or tissue material);
- patient's informed consent for genetic testing: signed consent for testing and personal data processing and (optionally) consent for storage and use of the sample in scientific research.

Transport requirements

Peripheral blood or bone marrow

Total timespan between sample collection and its receipt by the molecular laboratory should not exceed 24 hours at room temperature. In hot weather, material should be transported using lignin-wrapped cooling pads. The material should not be frozen. If it is necessary to store the material prior to transport, the sample must be stored at 2–8°C. Where transport within the recommended time-frame is impossible, the laboratory should be informed. When transporting biological material via courier companies, it is crucial to specify the maximum allowable time for delivery to the laboratory so that total time between collection and delivery does not exceed 24 hours.

Tissue biopsy

Before submitting the tissue sample, the laboratory should be informed. The saline-preserved sample should be delivered to the laboratory as soon as possible and preferably within 24 hours. Alcohol-fixed material can be submitted to the laboratory within seven days of collection.

Preliminary sample preparation

If the lymphocyte percentage in the test sample exceeds 50%, regardless of the absolute WBC count, there is no need to concentrate the material or to isolate peripheral blood mononuclear cells from bone marrow in a density gradient. If the lymphocyte percentage is below 50%, it is recommended to enrich the material in a density gradient or with an immunomagnetic method using anti-CD19 and possibly anti-CD5 in a two-step magnetic separation. Although this procedure is not mandatory, it may increase the diagnostic success rate, especially with the Sanger sequencing technique.

Test turnaround time

The test report should be available within three weeks from sample registration in the laboratory. In urgent cases, the laboratory should make arrangements for the report to be delivered within two weeks. In samples displaying interpretative difficulties, the reporting period may be extended to 4–6 weeks.

If a satisfactory result is not obtained after two diagnostic approaches (e.g. due to a lack of PCR product despite the use of different primer sets), the result is considered uninformative. In such cases, a second collection of material (e.g. bone marrow instead of peripheral blood) should be performed, or an alternative method (e.g. NGS sequencing instead of Sanger) should be applied.

Methodology

In accordance with the consensus recommendations of the ERIC panel of experts (European Research Initiative on CLL), the assessment of *IGHV* mutational status is based on the rearrangement amplification using 'Leader' primer mixtures. This technique is used in both Sanger sequencing and NGS methods.

Sequenced *IGHV*-D-J fragment should include the entire V-chain and the CDR3 region, vital for clonotype identification, hypermutation assessment, and subset classification. The standard size of the *IGHV* region analysed in typical cases ranges from 286 to 294 base pairs, depending on the identified clonotype.

This methodology should ensure a balanced amplification of all *IGHV* classes which allows for the spectrum of V-D-J clonotypes to be determined as broadly as possible, and minimises the risk of an uninformative result [8].

Sanger sequencing

Sanger sequencing, as the first technique used to assess somatic hypermutations, is recommended by the ERIC consortium as the gold standard for *IGHV* mutation status evaluation. The popularity of this method stems from its reliability and reproducibility, which made it the only technique available at the time the first guidelines were drawn up. Thanks to the long-standing use of this method, detailed guidelines have been developed, allowing standardised procedures of PCR reactions, interpretation and recording of results. The ERIC consortium additionally admits the laboratories performing *IGHV* status assessments to undergo a standardisation process and to be certified for the quality of their tests. The recommendations published in 2022 [8] emphasise that, despite the clear advantages, the NGS method needs to be standardised, especially in terms of the diagnostic significance of the results obtained. For example, the 98% cut-off, originally established for the Sanger method to distinguish between mutated and unmutated sequences, needs to be revised for NGS to guarantee diagnostic coherence. The first recommendations of the ERIC group, released in 2007 [9], contained guidelines concerning the type of genetic material used for the evaluation of the *IGHV* mutational status (peripheral blood collected on EDTA, DNA or cDNA), the temperature profile of the PCR reaction and the primer sequence (VH FR1, i.e. complementary to the FR1 fragment of the V variable), and the interpretation of the results thus obtained. At that time, a criterion was introduced to classify BcR IG sequences as unmutated or mutated sequences, defining a cut-off threshold of 98% concordance of the analysed sequence with the closest germline sequence.

Some caution was recommended in interpreting results when the concordance of the sequence with the germline sequence was 97.00–97.99%, classifying them as borderline mutated. This criterion is applied to both a result obtained with Sanger sequencing and with NGS sequencing, as no other cut-off threshold has been set for this method so far.

The guidelines were updated in 2017 [10] and again in 2022 [8]. The main amendments concerned the use of primers, known as ‘Leader Primers’, which replaced the VHFR1 primers. The new primers made it possible to amplify the entire *IGHV* sequence undergoing rearrangement, as they attach above the VHFR1 sequence. This allows a more reliable assessment of the somatic mutation status and eliminates the risk of misclassifying the tested sequence as unmutated, which can result from analysing a PCR product that is too short. The PCR reaction was designed as a multiplex assay. Primermix leader forward consists of a mixture of nine primers complementary to the six major variable sequence types, while primermix reverse contains a mixture of four primers complementary to the six JH sequence types. This arrangement enables easy first amplification, leading to a clonal PCR product. The use of a multiplex reaction also reduces the risk of obtaining PCR product(s) for non-cancerous clonotypes, which can be present in small numbers in the course of, for example, inflammatory or infectious diseases.

The use of multiplex PCR involves a two-step Sanger-type sequencing reaction. In the first step, initial information on the type of variable part is obtained with the use of a separate primer complementary to the JH part. Based on the result, a second sequencing reaction is performed using a primer specific for the detected variable part. This approach simplifies the screening step, as amplification can be performed in a single tube for each patient. However, such a procedure involves a significant increase in analysis time, as a full assessment of the *IGHV* mutation status only becomes possible after two sequencing steps have been performed. A disadvantage of the Sanger method is also the difficulty in identifying a productive clonal chain when allelic exclusion occurs. In such a case, the two clonal chains are amplified together and, for their identification, separate sequencing of both must be performed.

Next Generation Sequencing

The Next Generation Sequencing (NGS) technique is an array of diagnostic techniques implemented on different analytical platforms, rather than a single research method.

The Next Generation Sequencing using FR1 primers to determine *IGHV* mutation status was first used by Stamatopoulos et al. [11] in 2017. Over time, laboratories have

adapted the methodology to ‘Leader’ primers, achieving identity percentages similar to Sanger sequencing results. Commercial kits are now available to assess *IGHV* mutation status on ‘Leader’ primers on the Ion Torrent and Illumina platforms. It is also possible to perform the test using Illumina, Ion Torrent or Nanopore technologies as a laboratory developed test (LDT).

The critical requirement for NGS testing is that the range of sequencing should include the full leader sequence (>450 bp), comprising the CDR3 region and the full V-sequence of 286–294 base pairs. Correct bioinformatic analysis is also important. The result of the NGS testing is a list of clonotypes identified in the sample, including both leukaemic chains and polyclonal background derived from normal lymphoid cells. The role of the laboratory geneticist is to correctly distinguish between the pathological clonal chains, which are represented by most of sequencing reads, from the polyclonal background. This is not always a straightforward task. The presence of two chains with different amplification efficiencies (allelic exclusion effect, Figs. 1 A–C), subclonal tumour complexity, bi-clonality (Figs. 1 D–F), or low reaction efficiencies may cause problems as they can restrict the method’s ability to distinguish cancerous from non-cancerous clonotypes.

Taking into consideration these diagnostic difficulties, the laboratory should validate the in-house method or verify the CE-IVD method and specify distinct cut-off thresholds so as to properly determine the cancer clone. The laboratory should also implement quality control measures such as monitoring the total number of reads in the samples and the rates of uninformative and non-diagnostic results (definitions below).

Immunoinformatic analysis of clonotypes and mutational status

The ERIC group recommendations from 2017 incorporate requirements concerning the use of the IMGT/V-QUEST database (available at www.imgt.org) as the main tool for the analysis of the status of somatic mutations (SHM) of the examined sequence. It was also indicated as necessary to search for the stereotyped *IGHV* rearrangements, with the help of the ARResT/Assign Subsets database, available at https://bat.infospire.org/arrest/assign_subsets [13]. The IMGT/V-QUEST database (‘ImMunoGeneTics international system’) enables the evaluation of the status of the rearranged *IGHV* nucleotide sequence, submitted in the FASTA or FASTQ format, and to specify its closest germline sequence. This tool allows for analysis of the receptor sequences present on B and T lymphocytes in both humans and animals. The results of this analysis are presented as tabular data, and an example of a custom sequence analysis is set out in Figure 2.

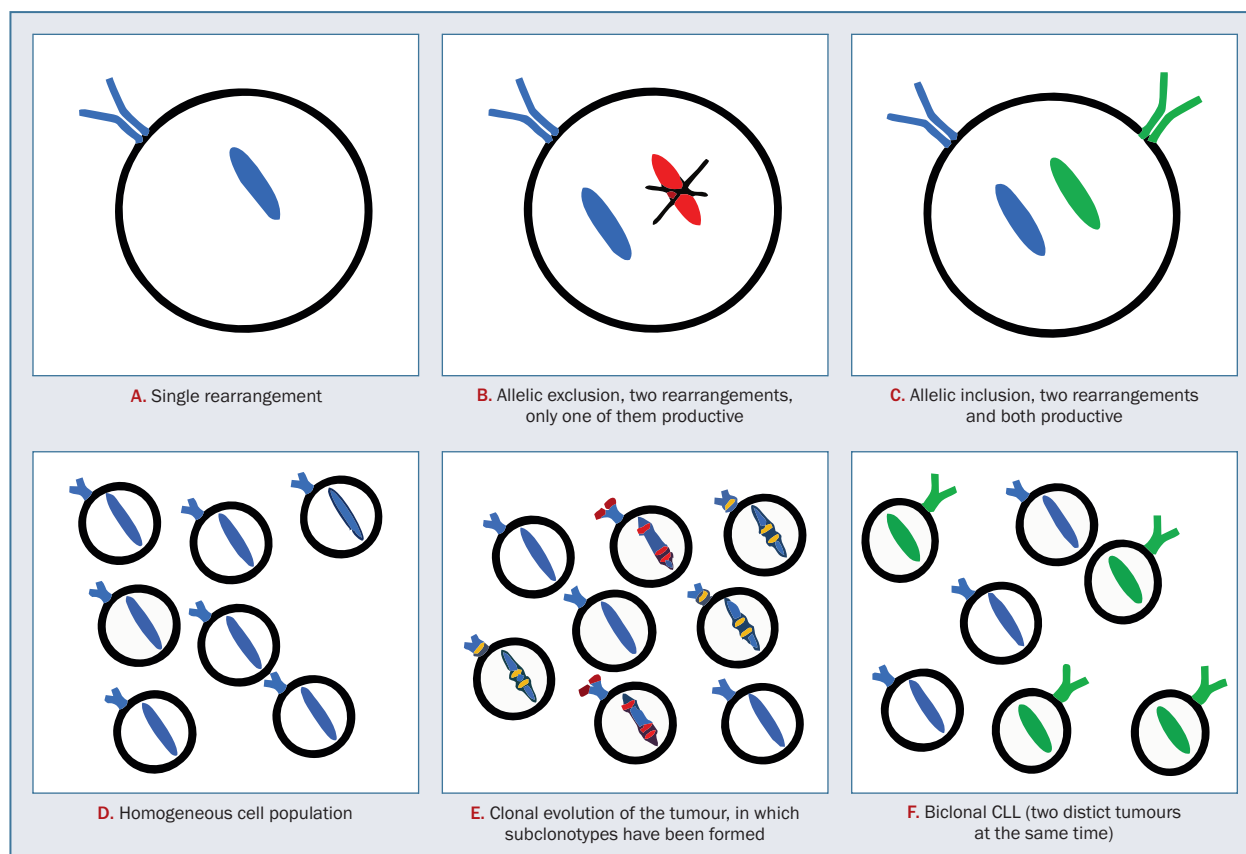


Figure 1. Co-occurrence of two clonal chains within a single tumour clone (A–C): in about 80% of cases V-D-J rearrangement results in a single productive sequence that allows expression of the normal BcR receptor (A). In about 20–30% cases, the rearrangement is unproductive. This phenomenon is known as allelic exclusion – it is driven by activation of a spare germline allele and rearrangement of the V-D-J. It results in a single productive sequence, which allows expression of the normal BcR receptor, and an additional unproductive sequence (B). Rarely, in situation of dysregulation of the rearrangement assembling, both alleles are activated and yield productive sequences. Then, two BcR receptors undergo coexpression. This phenomenon is referred to as allelic inclusion (C) [8].

Clonal heterogeneity of CLL (D–F): in some patients, a single, genetically homogeneous CLL cell population is present (A). However, after a prolonged disease progression, secondary mutations in the V-D-J sequences arise, and co-occurrence of genetically similar, but not identical, *IGHV* sequences, (derived from a single primary sequence), may be observed. Co-occurrence of two distinct populations of clonal lymphocytes (C) is very rare. These are often marked by different classes of *IGHV* sequences and may display different antigenicity upon cytometric examination (e.g. expression of both kappa and lambda light chains) [12].

The table generated by the IMGT/V-QUEST database contains the following information:

- **Productive IGH rearranged sequence (no STOP codon and in-frame junction)** – productive and rearranged sequence (without the STOP codon, in-frame junction, CDR3 sequence present);
- **V-GENE and allele J-GENE and allele D-GENE and allele** – the specification of the genes and alleles of V-D-J rearrangement; the nomenclature was approved by the Human Gene Nomenclature Committee with the support of the NCBI. In the presented case, the programme lists two variable sequences but is not able to determine which is the more likely. According to the guidelines, all genes and alleles found for the tested sequence should be displayed in the final report;
- **Identity** – the percentage of concordance (identity) with the closest germline sequence, which makes it possible to classify the tested sequence into the group of mutated or unmutated sequences.

If an unproductive sequence is detected, the database returns alerts. An example of such a result (based on an in-house analysis) is shown in Figure 3.

Apart from the obvious cases of detection of productive or unproductive sequences, there are also incomplete variations of the *IGHV* chain, defined as 'ORF' (Open Reading Frame). These are sequences that have a correct junction in the open reading frame, but do not display all the characteristics of a productive sequence. If the same sample does not have the other productive chain, the ORF variations should be treated as productive. According to the

Result summary: Consensus_784_3B	Productive IGH rearranged sequence (no stop codon and in-frame junction)		
V-GENE and allele	Homsap IGHV3-74*01 F, or Homsap IGHV3-74*03 F	score = 1206	identity = 90.97% (262/288 nt)
J-GENE and allele	Homsap IGHJ5*02 F	score = 156	identity = 78.43% (40/51 nt)
D-GENE and allele by IMGT/JunctionAnalysis	Homsap IGHD6-19*01 F	D-REGION is in reading frame 1	
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[25.17.38.11]	[8.8.19]	CVRDIHEGRGWFRGAGWFDPX (TRP 118 not identified)

Figure 2. Result of sequence analysis in IMGT/V-QUEST database (productive sequence)

Result summary: Consensus_969	Unproductive IGH rearranged sequence (stop codons, out-of-frame junction)		
V-GENE and allele	Homsap IGHV1-3*02 F	score = 1431	identity = 99.65% (287/288 nt)
J-GENE and allele	Homsap IGHJ4*02 F	score = 123	identity = 72.92% (35/48 nt)
D-GENE and allele by IMGT/JunctionAnalysis	Homsap IGHD3-3*01 F	D-REGION is in reading frame 2	
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[25.17.38.11]	[8.8.X]	CA*IRITIFGVVIEPVSXE#DYW
JUNCTION length (in nt) and decryption	74 nt = (5)- 6{3}+5(31)+2{18}-7(10)	(3'V)3'{N1}5'(D)3'{N2}5'(5'J)	

Figure 3. Result of sequence analysis in IMGT/V-QUEST database (unproductive sequence)

Result summary: Consensus_366	Nucleotide deletions have been detected (shown by dots in the alignments):				
	localization	nb of deleted nt	causing frameshift	from V-REGION codon	from nt position in user submitted sequence
	CDR2-IMGT	3	no	57	114
IMGT/V-QUEST results after filling the deletion(s) gap(s): Potentially productive IGH rearranged sequence (no stop codon and in-frame junction) (Check also your sequence with BLAST against IMGT/GENE-DB reference sequences to eventually identify out-of-frame pseudogenes) CLL subset #2 (however V-REGION partial in 5')					
V-GENE and allele	Homsap IGHV3-21*01 F, or Homsap IGHV3-21*02 F		score = 1099	identity = 94.29% (231/245 nt) [93.88% (230/245 nt)] - (V-REGION partial in 5')	
J-GENE and allele	Homsap IGHJ6*02 F		score = 193	identity = 84.91% (45/53 nt)	
D-GENE and allele by IMGT/JunctionAnalysis	Homsap IGHD1-1*01 F		D-REGION is in reading frame 1		
FR-IMGT lengths, CDR-IMGT lengths and AA JUNCTION	[11.17.38.11]		[8.7.9]	CATDRTGMDVW	
JUNCTION length (in nt) and decryption	33 nt = (11)+2{0}-1(4)-12{1}-17(15)		(3'V)3'{N1}5'(D)3'{N2}5'(5'J)		

Figure 4. Result of sequence analysis in the IMGT/V-QUEST database (sequence belonging to subset #2)

current guidelines, if unproductive sequences are identified, the status of the somatic hypermutation should not be derived from it, and such a result should not be reported, as the objective is to detect productive sequences. During the analysis, apart from the application of the programme default settings, it is useful to use advanced options such as ‘Search for insertions and deletions in V-region’. This is particularly important for sequences characterised by very low concordance with the germline sequence. This option makes it possible to omit the insertions or deletions in an analysed region, and thus to obtain an optimal match to the nearest germline sequence in the case of insertion-deletion

variants. An additional tool, available in the IMGT/V-QUEST advanced functionality group, is the option: ‘Clinical application: search for CLL subsets #2 and #8’, which allows the assignment of a clinically relevant subset. If the result is positive, a red highlighted message appears, e.g. CLL subset #2. By clicking the question mark icon, detailed information on the clinical relevance of the specific subset is displayed, with links to the specialised literature provided (Fig. 4). Before starting analysis in the IMGT/V-QUEST data-base in the case of the Sanger method, pairwise alignment programmes (e.g. BioEdit) should be used. They align the sequences obtained during bidirectional sequencing

readCount	readFract	targetSequences
38998.0	0.999359	TNACCATGGACTGGANNTGGAGGTTCTCTTTGTGGTGGCAGCAGCTACAGGTAAGGGGCTTCCTAGTCCTAAGGCTGAGGAAGGGATCTGGTTT
10.0	2.562591	GAGGTGAAGAAGCCTGGGGCCTCAGTGAAGGTCCTCGCAAGGCTTCTGGATACACCTTCACCACTTCTGATATCAACTGGGTGCGACAGGCCTCTG
4.0	1.025036	GACCTGCACCTTCTCTGGGTTCTCACTCAGCACTAGTGGAGTGGGTGGGCTGGATCCGTCAGCCCCAGGAAAGGCCCTGGAGTGGCTTGCACTC
3.0	7.687773	GTGCCGAAGTGCAGCTGGTGCAGTCCGGAGCAGAGGTGAAAAAGCCCGGGGAGTCTCTGAGGATCTCTGTAAGGGTTCTGGATACAGCTTTACCA
2.0	5.125182	GATTCATGGAGAAATAGAGAGACTGAGTGTGAGTGAACATGAGTGAGAAAACTGGATTGTGTGGCATTTTCTGATAACGGTGTCTTCTGTTTGC
2.0	5.125182	GGAGTCGGGCCAGGACTGGTGAAGCCTTCACAGACCTGTCCCTCACCTGCAGTGTCTCTGGTGGCTCCATCAGCAGTGGTAGTTACTACTGGAGCT
1.0	2.562591	TCCATCAGTAATGCTAATTACTTCTACGGCTGGATCCGCCAGCCCCACGGGGGGCCTGGAGTGGATTGGGAGTATTTACTATAGTGGGAGAACATC
1.0	2.562591	CCATATCAGTGGACACGTCCAAGAACCAGTTCTCCCTGGACCTAAAGTCTATGACGGCCGCGGACACGGCCGTATATTTTGTGCGGTGAGTGTGACC
1.0	2.562591	CCCCTGTGCTCTCCACAGGTGTCCAGTCCAGGTGCAGCTGGTGCAGTCTGGGGCTGAGGTGAAGAAGCCTGGGTCCTCGGTGAAGGTCTCTCTGC
1.0	2.562591	CCTGCAAGGCTTCTGGAGGCACCTTCAGCAGCTATGCTATCAGCTGGGTGCGACAGGCCCTGGACAAGGGCTTGAGTGGATGGGAGGGATCATCC

Figure 5. Example of NGS method *IGHV* sequencing result. The red box indicates the clonal chain sequence, which accounts for more than 99% of all NGS reads

using the forward and reverse primers. Only a sequence prepared in this way may be subjected to further IT analyses. For a correct analysis, after pasting the nucleotide sequence into IMGT, add the fastq format header '>name' before the start of the sequence.

For NGS methods, the result is presented in a tabular form. The following rows correspond to the identified chains ranked in descending order of abundance. In individual columns, parameters such as the number of reads (in the MIXCR software example above, called **readCount**), the percentage of each chain (here: **readFraction**), and the nucleotide sequence (**targetSequences**) are presented (Fig. 5). Sequences from this column can be pasted directly into the IMGT/V-QUEST software. However, some programmes insert additional special characters into the sequence which must be removed before starting the analysis. The fastq format header '>name' must also be added before starting the analysis.

CLL subsets analysis

ARResT/Assign Subsets database is the source of information about the BcR IG receptor stereotypy; this is a bioinformatics tool recommended by the ERIC Expert Panel for assigning specific CLL subsets. When entering sequences into the database, it is important to ensure that the nucleotides are not ambiguously defined (according to IUPAC codes, e.g. R, W or N). If they are, the database will not assign a subset (Fig. 6). However, if such a problem occurs, the nucleotide can be changed manually to adenine 'A', which has the least impact on the analysis result. Subset assessment involves the confidence statistics of the match ('confidence'). Only subset results with the status of 'average', 'high' or 'extreme' are eligible for reporting. 'Low' and 'borderline' statuses indicate an uncertain outcome, and should not be included in the reports.

A subset defines a patient group characterised by a homogeneous biology of disease progression, its clinical manifestation, and the response to therapy. Subsets can be assigned for approximately 40% of CLL patients and

are a prognostic factor independent of the SHM status. An analysis of more than 29,000 CLL cases revealed the presence of 29 subsets based on CDR3 sequence homogeneity [12]. However, from a clinical perspective, only two subsets are relevant: CLL#2 and CLL#8, which, according to the 2022 recommendations, should be included in the final report [8]. Subset CLL#2 is associated with an aggressive course of the disease and includes patients with the *IGHV3-21/IGLV3-21* variable sequence and the characteristic CDR3 sequence in the VH and VL regions. Not every *IGHV3-21* sequence belongs to the CLL#2 subset, because the specific structure of the CDR3 part is crucial. Although the *IGHV* mutated status is found in the majority of patients, and is generally associated with a good prognosis, the CLL#2 subset is a negative prognostic factor independent of the *IGHV* mutation status. In such cases, patient management should depend on the presence of the CLL#2 subset. In addition, the CLL#2 subset is associated with an increased frequency of mutations in the *SF3B1* gene and aberrations (deletions and mutations) involving the *ATM* gene (Fig. 7).

The CLL#8 subset indicates an aggressive disease course, and includes patients with altered *IGHV4-39/IGKV1(D)-39* genes characterised by specific VH and VK CDR3 sequences. The clonotype classification indicates that *IGHV4-39/IGKV1(D)-39* belongs to a group of unmutated rearrangements. Patients classified as CLL#8 have a significantly higher risk of Richter's transformation (RT). Moreover, chromosome 12 trisomy and *NOTCH1* gene mutation are frequently observed in these patients.

Although the current recommendations do not require reporting of the CLL#4 subset, it should be noted that the CLL#4 subset (*IGHV4-34*) is associated with an indolent clinical course and is typical in young adult patients.

The update of the recommendations published in 2022 pays special attention to results that are difficult to interpret (Tab. I) [8].

In 2024, there was a further update to the guidelines on reporting the results of the SHM status (an example is shown in Figure 8) [14]. The ERIC group commented on the

Cite us!

Assigning new members to existing subsets of stereotyped antigen receptor sequences, currently applicable to the 19 major subsets of stereotyped B-cell receptors in chronic lymphocytic leukemia (CLL).
10.11.19 powered by ARResT/SeqCure; ARResT/Subsets; IMGT/V-QUEST; IMGT/CLL-DB

ARResT | cite us | news | help | contact us | BAT cave

Please consider using Chrome / Firefox / Safari for best viewing and full functionality.

Your antigen receptor sequences:
provide up to 50 FASTA-formatted FULL NUCLEOTIDE IG sequences. Check example below, ~100kb upload limit.
Copy-paste or type (?) up to 50 properly formatted nucleotide sequences here.

Wybierz plik

Nie wybrano pliku

Clear browsed file

Or click to load example
FASTA

DISCLAIMER:

There is no guarantee that ARResT/AssignSubsets will be able to properly assign all your sequences to subsets, please bear this in mind when making decisions, especially important ones on e.g. clinical care, and especially with "borderline-" or low-confidence assignments. To help us improve ARResT/AssignSubsets, please contact us.

Assign to Subsets

or

reset

Citation and acknowledgements:

We're now published in Bioinformatics:
ARResT/AssignSubsets: a novel application for robust subclassification of chronic lymphocytic leukemia based on B cell receptor IG stereotypy. Vojtech Bystry, Andreas Agathangelidis, Vasilis Bikos, Lesley Ann Sutton, Panagiotis Baliakas, Anastasia Hadzidimitriou, Kostas Stamatopoulos, Nikos Darzentas.
Bioinformatics 2015]

Figure 6. View of the ARResT database with the window for entering the IGHV sequences in FASTA format

Result summary: 215		Productive IGH rearranged sequence (no stop codon and in-frame junction (a)											
V-GENE and allele		Homsap IGHV3-21*01 F, or Homsap IGHV3-21*02 F	score = 797	identity = 85.25% (185/217 nt)									
J-GENE and allele		Homsap IGHJ4*02 F	score = 87	identity = 64.58% (31/48 nt)									
D-GENE and allele by IMGT/JunctionAnalysis		Homsap IGHD3-22*01 F	D-REGION is in reading frame 2										
FR-IMGT lengths and AA JUNCTION		[1.17.38.11]	[8.8.20]	XARVLXXPYYYDSXGLXPGGY X (2nd-CYS 104 and TRP 118 not identified)									
label [+ heat map, if appl.]	SeqCure	subset	confidence	score									
215	note	CLL#2	high	71,2									
		<table><tr><th>Parameter</th><th>Characteristics</th></tr><tr><td>Clinical course</td><td>Aggressive</td></tr><tr><td>Mean TTF in years</td><td>1,9 (0-7,9)</td></tr><tr><td>Accompanying genetic alterations</td><td>SF3B1 36-45%, ATM 26%, TP53 0-5%, TP53del 0-4%</td></tr></table>				Parameter	Characteristics	Clinical course	Aggressive	Mean TTF in years	1,9 (0-7,9)	Accompanying genetic alterations	SF3B1 36-45%, ATM 26%, TP53 0-5%, TP53del 0-4%
Parameter	Characteristics												
Clinical course	Aggressive												
Mean TTF in years	1,9 (0-7,9)												
Accompanying genetic alterations	SF3B1 36-45%, ATM 26%, TP53 0-5%, TP53del 0-4%												

Figure 7. Example of subset #2 assignment

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Table I. Update of the recommendations of the ERIC group from 2022 [8]

Issue (testing standard)	Recommendations
Methodology	The report must contain information about the type of the starters applied, the method of PCR product analysis, sequencing method and the tools used for bioinformatics analysis for the evaluation of the SHM status and stereotypies
Identification of IGH genes and alleles	The report must contain information about all identified genes and alleles
Functionality	The evaluation of the SHM status is carried on solely on the basis of productive rearrangements. Detection of only unproductive rearrangements (which is extremely rare) requires a reason for the unproductivity (e.g. IG pseudogene, STOP codon, out-of-frame splicing, large indel)
<i>IGHV</i> gene: percentage of nucleotide match to the germline sequence given to two decimal places according to IMGT	Classification: • unmutated $\geq 98.00\%$ • mutated $<98.00\%$ • borderline mutated 97.00–97.99%
Subset identification/BcR IG stereotypy	It is recommended to provide information on subset affiliation with well-defined clinical relevance (currently CLL #2 and CLL #8 subsets)
Issue (difficult cases)	Recommendations
Single unproductive rearrangement	It is recommended to repeat the PCR reaction with an alternative primer set or using cDNA or to perform an NGS assay to obtain detailed information on clonal architecture
Double rearrangements: one productive and one unproductive	As in the standard case. SHM status is to be determined from the productive rearrangement independently of the SHM status of the unproductive sequence (which is not reported because it does not contribute any clinically relevant information)
Double productive rearrangements — compatible SHM status	As in the standard case. SHM status to be specified as mutated or unmutated depending on the SHM status
Double productive rearrangements — incompatible SHM status	Check the immunophenotype result for the presence of two clonal populations. It is recommended to tell the attending physician that it is safer to treat this case as unmutated and perform a follow-up assessment
More than two productive rearrangements	The immunophenotype result should be checked for the presence of two or more clonal populations. It is recommended to perform an NGS test in order to determine the relative frequency of each clonotype and to select the dominant clonotype
No anchoring amino acids C104/W118	SHM assessment can be performed if IG expression is present on leukemic cells and/or the G-X-G motif within the VH FR4 is conserved

need to include information on the relationship between somatic hypermutation status and response to treatment in their report (Tab. II).

Report preparation

Mandatory information to be provided with an *IGHV* test report:

- patient identification data, ordering clinicians identification data, and laboratory data. Laboratory performs tests in accordance with the Act on Laboratory Medicine of 15 September 2022 [19];
- biological sample data, data of person performing and authorising test, and pagination on each page of report (following the Regulation of the Minister of Health of 6 April 2020 on types, scope and models

of medical records and the manner of their processing) [20];

- methodology and bioinformatics analysis description. Information common to both methods:

- type of genetic material used as a template for the PCR: gDNA or cDNA;
- type of primers used in the reaction — in accordance with the ERIC Leader group guidelines;
- sequencing method used: Sanger (bidirectional) or NGS;
- bioinformatics tools used to analyse the obtained sequences: IMGT/V-QUEST, ARResT/assignSubset. According to Molecular Haematology Section of the PTGC recommendations, a second Ig BLAST informatics tool is recommended for verification, with the proviso that IMGT/V-QUEST is superior.

Table II. Update of the 2024 ERIC group recommendations [14].

Result	Interpretation
Mutated (example from Fig. 8)	Following the 98% germline identity cut-off, which is used for discrimination CLL cases into the <i>IGHV</i> -mutated or <i>IGHV</i> -unmutated category, this case belongs to <i>IGHV</i> -mutated category which is generally associated with a favourable prognosis
Unmutated	Following the 98% germline identity cut-off, which is used for discrimination CLL cases into the <i>IGHV</i> -mutated or <i>IGHV</i> -unmutated category, this case belongs to <i>IGHV</i> -unmutated category, which is associated with an unfavourable prognosis, poorer response to chemoimmunotherapy and shorter progression-free survival when treated with a combination of Venetoclax and anti-CD20 antibodies, compared to CLL cases in the mutated category [14]
Borderline mutated	Following the 98% germline identity cut-off, which is used for discrimination CLL cases into the <i>IGHV</i> -mutated or <i>IGHV</i> -unmutated category, this case belongs to <i>IGHV</i> -mutated category. The result shows concordance with the germline sequence close to the 98% value. This case is therefore included in the borderline mutated CLL group. Such cases require a lot of caution with regard to the exact prognostic implications
Subset #2	Following the 98% germline identity cut-off, which is used for discrimination CLL cases into the <i>IGHV</i> -mutated or <i>IGHV</i> -unmutated category, this case belongs to <i>IGHV</i> -mutated category. This particular rearrangement belongs to stereotypic subset #2, which is associated with an unfavourable prognosis, a poorer response to chemoimmunotherapy, regardless of the somatic hypermutation status [15, 16]. The predictive value for patients belonging to subset #2 treated with targeted therapies is currently not defined
Subset #8	Following the 98% germline identity cut-off, which is used for discrimination CLL cases into the <i>IGHV</i> -mutated or <i>IGHV</i> -unmutated category, this case belongs to <i>IGHV</i> -unmutated category. This particular rearrangement belongs to stereotypic subset #8, which is associated with the highest risk of Richter's transformation of all CLL cases [17]

Information required only for the NGS method:

- name of assay used, manufacturer and scope of application (for commercial methods);
- threshold number of reads or contigs obtained;
- bioinformatics analysis: software (name, manufacturer, version) and additional software tools used (optional);
- full characteristics of productive clonotypes (V-D-J class and subclass) and, in the case of several subclasses, listing them (e.g. V1-69 or V1-69D). The assessment of rearrangements includes identification of all genes and alleles: *IGHV*, *IGHD*, *IGHJ*; it is acceptable to report rearrangements without identification of *IGHD*, but only in difficult cases;
- percentage of nucleotide match to the germline sequence accurate to two decimal places;
- description of mutation status: (mutated <98%/unmutated ≥98%/borderline mutated 97.00–97.99%); the record of the SHM status assessment on the report must be in Polish, without the use of acronyms or English-language loanwords;
- characterization of the CLL subset, if identified (based on international recommendations, only subset CLL#2 and subset CLL#8 are reported); in addition, reports in which subset CLL#2 is identified should include information that it is a prognostically unfavourable

change, regardless of *IGHV* status. Currently, there is no evidence of the predictive value of subset CLL#2 in relation to targeted therapies [13].

Crucial to the result is correct interpretation – it should indicate the following aspects:

- determining whether the sequence is productive or unproductive (unproductive sequences have no proven clinical significance and therefore are not reported in the result);
- result must be identified as mutated/unmutated/ /borderline mutated, and information concerning the association with better/worse prognosis treated with caution concerning prognosis assessment;
- report should indicate the ranges of homology with the germline sequence, which form the basis for interpreting the result;
- Adding a source to the interpretation in the form of a publication: Immunoglobulin gene sequence analysis in chronic lymphocytic leukaemia: the 2022 update of the recommendations by ERIC, the European Research Initiative on CLL Leukaemia (2022) 36: 1961–1968; <https://doi.org/10.1038/s41375-022-01604-2>;
- subsets with verified clinical relevance (#2 and #8 according to the ERIC group) must be identified and included in the report;

1. Example of the IG report, *IGHV*-mutated

Name of the Hospital/Laboratory

Determination of *IGHV* gene SHM status

Date of result 22/01/2022
Date of sample collection 09/01/2022

Patient name ***
Diagnosis CLL
Tissue type blood
Molecule type genomic DNA

Utilized methodology
PCR amplification of *IGHV*-*IGHD*-*IGHJ* gene rearrangements with leader primers
Genescan analysis
Bidirectional Sanger sequencing [or next-generation sequencing (whichever applies)]
Immunoinformatics analysis IMGT/V-QUEST [or any specific software for NGS-based IG gene analysis (whichever applies)]

Result
A productive *IGHV*3-2301/*IGHD*4-1701/*IGHJ*402 gene was detected
The rearranged *IGHV* gene had 96.2% nucleotide identity with the germline sequence of the *IGHV*3-2301 gene

Interpretation
Following the 98% germline identity cut-off which is used for discriminating CLL cases into the *IGHV*-mutated or *IGHV*-unmutated category, this case belongs to the *IGHV*-mutated category which is generally associated with favorable prognosis

Signatures]

Figure 8. Examples of SHM status assessment results [14] (explanation in Table II)

- providing their prognostic significance, a list of publications and an indication that subset #2 is an additional prognostic factor independent of mutation status. If the detected rearrangement does not belong to a subset of proven clinical significance, no additional information should be included in the report.

Certification of *IGHV* mutational status

On account of the crucial importance of maintaining high quality of the *IGHV* mutation status assessment, it is important to obtain the ERIC group certification or an alternative certification confirming the high standard of testing performed by the laboratory concerned. Participation in the ERIC group certification is voluntary and free of charge with the first application. To join the process, an online form must be completed and submitted (<https://ericll.org/ig-network/>). Two standardisation rounds are conducted each year — one based on DNA and the other on RNA/cDNA. The laboratory receives five test samples to analyse. The test should be performed according to the protocol recommended by the ERIC group. The result should be returned in the format indicated on the website and reports must include the following:

- identified genes and alleles (*IGHV*/*IGHD*/*IGHJ*);
- primers used;
- sequencing method;
- percentage of homology with germline sequence;
- rearrangement type (productive or unproductive);
- bioinformatics tools used for analysis;
- interpretation of the result.

The scope of information in the certification sample reports follows the recommended way of reporting the result in standard diagnostic samples described in these recommendations. If the test samples have been correctly assessed, the laboratory receives a certificate which is valid for three years from the date of issue. After this period, a laboratory can apply for renewal of the certificate, e.g. through GenQA, an institution which collaborates with ERIC and standardises *IGHV* mutation assessment testing based on principles that comply with its guidelines. GenQA certification is chargeable, takes place once a year, and involves the analysis of three test samples. The submitted results should be conforming to the European guidelines for reporting results, the ERIC recommendations, and the ISO15189 standard. The method and scope of reporting of certification samples in GenQA is very similar to ERIC certification requirements.

Examples of interpretations to be used for report

Example 1. Mutated *IGHV*

The detected rearrangement of the *IGHV* gene is productive and manifests ...% homology with *IGHV* germline sequences... It belongs to mutated sequences which indicate a good prognosis.

- Unmutated: germline concordance $\geq 98\%$;
- Mutated: germline concordance $< 98\%$;
- Borderline mutated: germline concordance 97.00–97.99%.

Example 2. Unmutated *IGHV*

The detected rearrangement of the *IGHV* gene is productive and manifests ...% homology with *IGHV* germline sequences... It belongs to unmutated sequences which indicate an unfavourable prognosis and poorer response to chemo(immuno)therapy and shorter progression-free survival in the case of therapy combining Venetoclax and anti-CD20 antibodies, compared to CLL cases belonging to mutated category [12, 14].

- Unmutated: germline concordance $\geq 98\%$
- Mutated: germline concordance $< 98\%$
- Borderline mutated: germline concordance 97.00–97.99%

Example 3. Borderline mutated

The detected rearrangement of the *IGHV* gene is productive and manifests ...% homology with *IGHV* germline sequences... The percentage of homologies fits within the range of borderline results (97.00–97.99%), and therefore this particular rearrangement is classified as a borderline mutated result. Caution is recommended in prognosticating, in spite of formal classification of the results into the mutated group.

Example 4. Subsets

The detected rearrangement belongs to subset #2 and has a negative prognostic significance, which is a prognostic factor independent of the mutation status of the *IGHV* chain.

Informative result

The evaluation of the *IGHV* mutational status is defined as the percentage of the identity between the sequence identified in CLL cells and the closest germline sequence based on the pattern in the IMGT/V-QUEST database. Such an assessment can only be made on the basis of sequences of a productive nature. Full identification of the *IGHV*, *IGHD*, *IGHJ* genes and alleles should also be included in the report. It is acceptable to report rearrangements without identification of *IGHD*, but only in difficult cases.

The mutation status can be ascribed to the three categories on the basis of the cut-off threshold value of 98%:

- Mutated: germline concordance $< 98\%$;
- Borderline mutated: concordance fits within the range 97.00–97.99%;
- Unmutated: germline concordance $\geq 98\%$;

The above information should be included in the report, entering the status (mutated/unmutated/borderline mutated) and the percentage of concordance with the germline sequence. An update to the ERIC group's recommendation for reporting unmutated cases was published

in 2024 [14]. This update is based on the availability of new targeted therapies for this group of CLL patients. ERIC proposes that the final report, in the case of unmutated *IGHV* genes, should also mention that these patients show a shorter disease progression-free survival compared to cases with *IGHV* mutation [13].

Non-informative result

This type of result is declared when it is impossible to assess the mutation status, *i.e.* when only a non-functional/unproductive chain was obtained or only a polyclonal background was obtained (no monoclonal sequence). The reason for the missing result (unfunctional/unproductive chain: out of reading frame and/or STOP codon) should be noted in the report. An alternative action may be suggested, such as performing an NGS test.

Non-diagnostic result

When a clonal PCR product or NGS result cannot be obtained due to technical or biological reasons (e.g. degradation of the material in the sample), a second sample collection is recommended. If the situation recurs, no further attempt is recommended. Complete withdrawal from test performing, or a refusal to accept the sample, must be reported to the attending clinician and these should be based on objective reasons, e.g. change in clinical diagnosis, deep remission, absence of clonal lymphocytosis in the sample (in MBL or SLL), absence of patient consent for the test or withdrawal of previously given consent. The above information should be included in the report.

Article information and declarations

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The authors declare no conflict of interest.

Declaration of data availability

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Benefits of infusion pump technology in treatment of patients with acquired hemophilia A and hemophilia A with inhibitor a Polish pilot observational study

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Abstract

Introduction: The authors compared the efficacy, practicality and personnel preference of the infusion pump method against those of bolus administration of rFVIIa.

Material and methods: Three patients with hemophilia A received recombinant factor VIIa infusion pump treatment. Their hemostasis and response to treatment was strictly monitored. Nursing personnel (n = 20) were surveyed on their preference and opinion regarding the infusion pump compared to bolus administration of recombinant factor VIIa.

Results: The maintenance of hemostasis was satisfactory in the group of patients who were administered recombinant factor VIIa via infusion pump, and no dosage was missed or delayed. A large majority of the surveyed personnel (75%) evaluated the infusion pump as offering a more comfortable and easier administration method than 4-hourly boluses.

Conclusions: Recombinant factor VIIa via infusion pump is more effective in maintaining hemostasis and is the preferred administration method among nursing personnel. Patients and personnel alike benefit from infusion pump administration of recombinant factor VIIa for hemophilia A.

Keywords: hemophilia A, acquired hemophilia A, inhibitor, recombinant factor VIIa, infusion pump

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Introduction

Hemophilia A (HA) is a congenital or acquired bleeding disorder characterized by deficiency or dysfunction of coagulation protein factor VIII (FVIII). Congenital hemophilia A (HA) is a recessive genetic disease of adult males and boys caused by mutation in the F8 gene on the X chromosome [1]. Acquired hemophilia A (AHA) is a very rare bleeding disorder, with an estimated incidence of 1.48 per million

per year [2]. Unlike HA, AHA is an autoimmune disease caused by spontaneous production of autoantibodies against clotting factor VIII (FVIII), and it occurs in both men and women [3]. The typical clinical manifestation of AHA includes sudden onset of bleeding in a person with no personal or family history of hemorrhages [4, 5]. AHA is a severe hemorrhagic disorder manifesting with bleeding events of differing severities. In contrast to HA, there is no strict relationship between the activity of FVIII in plasma

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Institute of Haematology and Transfusion Medicine.

and the severity of hemorrhagic diathesis in AHA patients [6]. About 30% of AHA patients initially experience minor bleeding, while others can suffer from life-threatening hemorrhages [3].

The most common symptoms of HA include muscle hematomas and intra-articular bleeding, while those of AHA include subcutaneous hemorrhages, mucosal bleeding, retroperitoneal bleeds, and prolonged bleeding after surgical procedures [1–3].

In more than 30% of patients with severe HA (defined as FVIII activity of less than 1 international unit (IU)/dL) and 3–13% of patients with moderate and mild hemophilia (FVIII activity of 1–5 IU/dL and >5–40 IU/dL respectively), inhibitor to FVIII will develop [7]. Inhibitors are alloantibodies to FVIII that typically neutralize factor VIII activity and thus also the function of infused clotting factor concentrates [8]. The occurrence of FVIII inhibitor currently represents the most serious complication of hemophilia A [9]. In the presence of these inhibitors, bleeding generally does not respond to replacement therapy using concentrates of the deficient clotting factor [7].

The treatment of HA typically involves regular infusions of factor VIII concentrates to prevent bleeding episodes, with doses adjusted based on the severity and individual responses [10]. The therapeutic approach for AHA is based on three key principles: stopping the bleeding, initiating immunosuppressive therapy to eliminate the inhibitor, and addressing any underlying condition identified [11]. The most effective treatment of AHA-related bleeding is the administration of by-passing agents (BPAs) such as recombinant activated factor VII (rFVIIa), activated prothrombin complex concentrate (aPCC), or recombinant porcine factor VIII (rp-FVIII) [12]. BPAs are also indicated in HA in patients with inhibitor titers higher than 10 Bethesda Units (BU)/mL, along with immune tolerance induction (ITI) [13].

rFVIIa (eptacog alpha) is given at a dose of 90 µg/kg every 2–3 hours as a 2–5 minute intravenous bolus. The interval between doses can be extended to 4, 6, 8 or 12 hours based on the severity of the bleeding [6, 14]. The frequency of bolus injections is a result of the short half-time of rFVIIa, which is 2.8 hours *in vivo* [12]. There have been studies discussing the use of an infusion pump instead of bolus injections in HA patients [15–17]. According to the rFVIIa Summary of Product Characteristics (SmPC), the product remains stable for 24 hours at 25 °C when stored in a 50 mL polypropylene syringe. This stability allows for intensive therapy with automatic rFVIIa boluses delivered via an infusion pump [6, 14].

Continuous infusion, as opposed to repeated bolus injections, is a more precise and time-efficient method of administration. It has the potential to reduce the burden on nursing staff and improve the patient's quality of life (QoL), as well as to reduce the overall cost of treatment [13, 14].

In this study, the authors compare the efficacy and practicality of, and personnel preference for, the infusion pump method against those of bolus administration of rFVIIa.

Aim of study

Comparison of efficacy and practicality of, and personnel preference for, infusion pump method versus those of bolus administration of rFVIIa.

Material and methods

The authors conducted a single-center prospective comparative study at the General Hematology Department, Copernicus Memorial Hospital, Lodz, Poland.

Patients

The study included patients with hemophilia A acquired ($n = 2$) or with inhibitor ($n = 1$) who received treatment with recombinant factor VIIa via an infusion pump. The efficacy of the treatment was assessed by monitoring patients' response to treatment and their adverse events profile.

Infusion pump

All three patients were administered eptacog alpha at dosage of 90 µg/kg. The drug was administered through a peripheral access on the arm. Prior to administration, patient weight was assessed and bolus intervals were set. The drug was not diluted and was kept in a 50 mL polypropylene syringe for no longer than 24 hours at room temperature.

Nurse evaluation

Highly-qualified nursing personnel who administered rFVIIa via infusion pump at the Department of General Hematology in Lodz ($n = 20$) were questioned on their preference and opinions regarding infusion pump compared to bolus administration of recombinant porcine factor VIII. This questionnaire consisted of seven questions (see Tab. I).

Results

Study group characteristics and patient outcomes

The study group consisted of three patients, two with AHA and one with HA with inhibitor. Detailed characteristics of the study cohort are set out in Tables II and III.

The authors present below case reports of the three patients who received rFVIIa infusion pump treatment.

Patient 1

A 74-year-old Caucasian female was referred to the Department of General Hematology in Lodz from a county hospital with suspicion of AHA, where she had been hospitalized due to sepsis. On admission, the patient had extensive

Table I. Questionnaire

Question 1	IV bolus administration every 4 hours	25%
Which method of administering hemostatic drugs (eptacog alpha, susoctocog alpha) do you think is more comfortable?	Supply in infusion pump replaced every 24 hours (boluses delivered automatically by pump)	75%
Question 2	1	35%
How would you assess the level of difficulty in the administration of recombinant porcine factor VIII concentrate as an intravenous bolus on a 24-hour basis?	2	15%
(1 = very easy, 5 = very difficult)	3	20%
	4	15%
	5	15%
Question 3	1	30%
How would you assess the level of difficulty in the supply of recombinant factor VIIa in an infusion pump replaced every 24 hours?	2	25%
(1 = very easy, 5 = very difficult)	3	15%
	4	15%
	5	15%
Question 4	Need for frequent administration at precise time intervals	20%
What do you think is the greatest difficulty in the administration of recombinant porcine factor VIII concentrate in an intravenous bolus?	Frequent blood sampling	30%
	Large number of vials administered at one time	35%
	Long preparation time	15%
	Other	0%
Question 5	Permanent connection of patient to infusion pump	55%
What do you think is the greatest difficulty in the administration of recombinant factor VIIa in an infusion pump?	Preparing medicine in syringe for infusion pump	20%
	Long preparation time	15%
	Other	10%
Question 6	Shorter daily duration of drug administration to patient	15%
What do you think is the biggest advantage of administering recombinant factor VIIa in the infusion pump?	Administering drug at precise time intervals	65%
	No need for frequent blood collection	10%
	Short preparation time	10%
Question 7	Possibility of monitoring concentration of factor	15%
What do you think is the biggest advantage of administering recombinant porcine factor VIII concentrate as an intravenous bolus?	Drug administration without use of an infusion pump	40%
	Patient is not permanently connected to infusion pump	45%

subcutaneous ecchymoses on the upper and lower limbs and lateral sides of the thorax. Laboratory studies revealed a prolonged activated partial thromboplastin time (aPTT) of 104.9 s (normal range 25.4–36.9 s). The level of factor VIII was less than 1% and inhibitor titer against human factor VIII was 4.9 j.B/mL (normal < 0.5 j.B/mL). The patient was started on treatment with eptacog alpha (0.09 mg/kg intravenously (i.v.) via an infusion pump with bolus intervals of three hours) and eradication of inhibitor via the use of prednisone

(1 mg/kg per day). The treatment course was uneventful, and the rFVIIa administration was prolonged firstly to 4-hourly and then to 6-hourly intervals. The treatment was then followed by rpFVIII. Due to the development of anemia and increased demand for rpFVIII, aPCC was administered. The inhibitor eradication therapy was intensified with cyclophosphamide and later rituximab, resulting in elimination of the inhibitor and an increase in FVIII level. The treatment course, as well as the levels of inhibitor titer and FVIII, are depicted in Figure 1.

Table II. Characteristics of study group

Analyzed trait	Value (number of patients)
Type of hemophilia	
Acquired hemophilia A	2
Hemophilia A with inhibitor	1
Age at beginning of pump treatment	
50–60	1
61–70	0
71–80	1
81–90	1
Sex	
Female	1
Male	2
Disease at beginning of pump treatment	
New diagnosis	2
Relapse	1
Lines of treatment in this hospitalization	
1	0
2	2
3	1
Previous treatment	
Recombinant factor VIIa	2
Porcine factor VIII	1
Activated prothrombin complex concentrate	3
Inhibitor eradication	
Prednisone, rituximab, cyclophosphamide	2
No treatment	0
Bleeding score at diagnosis	
1	1
2	1
3	0
4	0
5	1
Bleeding score at beginning of treatment	
1	2
2	0
3	1
4	0
5	0

Table III. Laboratory parameters of study cohort

Laboratory parameters at beginning of pump treatment (value)	Value		
	Patient 1	Patient 2	Patient 3
Factor VIII level [%]	1%	<1%	<1%
Inhibitor titer (BU)	4.9	1,239	1
PLT ($\times 10^3/\mu\text{L}$)	343	226	204
APTT (s)	105	118	87
PT (s)	1.04	1	0.96
WBC ($\times 10^3/\mu\text{L}$)	13	10	10
HGB (g/dL)	8.3	9.8	8.7

Table IV. Comparison of infusion pump and bolus administration

Feature	Bolus administration	Infusion pump administration
Frequency of administration	Every 4 hours	Replaced every 24 hours
Precise time of administration	Depends on nursing staff	Depends on pump settings
Precise dosage administration	Impossible	Available
Time spent on administration	90 min/day	15 min/day
Iatrogenic sleep deprivation	Present	Not present
Burden on nursing staff	Heavy	Mild

Patient 2

An 86-year-old Caucasian male was admitted with extensive subcutaneous ecchymoses on both upper limbs and a suspicion of AHA. His laboratory studies revealed a greatly prolonged aPTT of 129 s (normal range 25.4–36.9 s), factor VIII level of less than 1%, and inhibitor titer of 1,331 BU/mL. During hospitalization, hemostatic treatment was administered. Initially this took the form of recombinant factor VIIa using an infusion pump, then aPCC, resulting in disappearance of the symptoms of bleeding diathesis. At the same time, inhibitor eradication treatment was used (prednisone, cyclophosphamide, rituximab), achieving an improved clinical condition, a reduction of the inhibitor titer to 140 BU/mL, and an increase of FVIII level up to 26%. The patient's treatment course is depicted in Figure 1.

Patient 3

A 56-year-old male with severe HA was admitted after an episode of extensive spontaneous bleeding into the subcutaneous tissue of the thorax and pleural cavities.

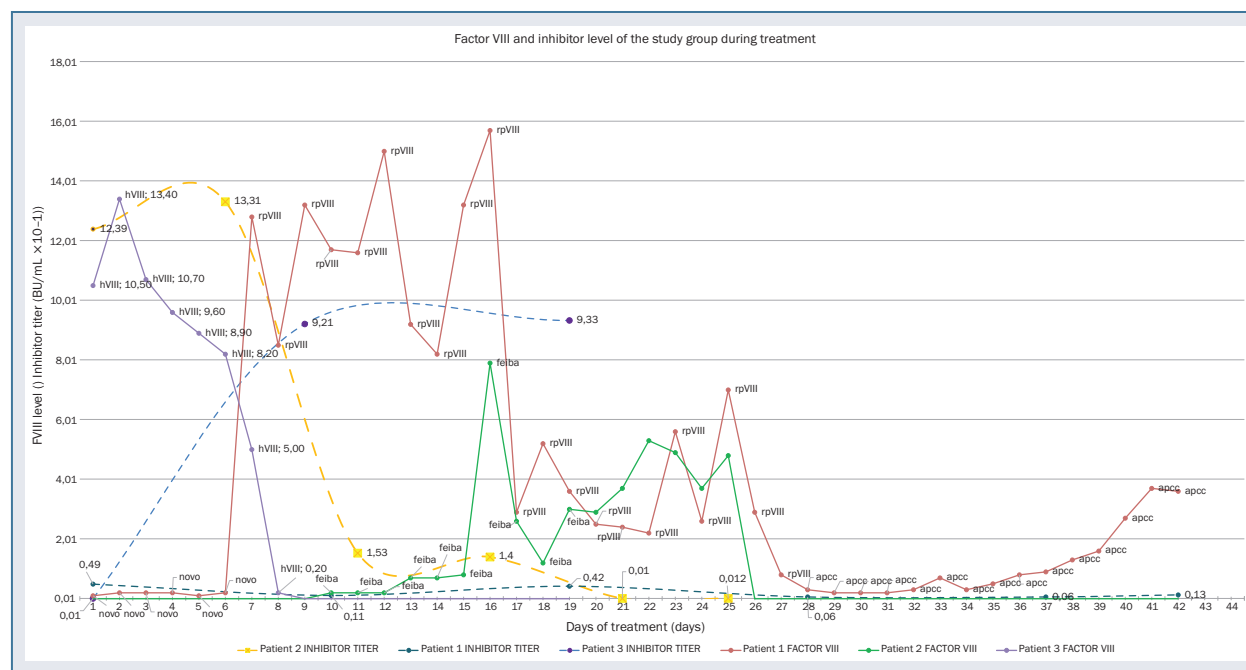


Figure 1. Laboratory parameters (factor VIII level, inhibitor titer) and course of treatment of study group (n = 3). APCC — activated prothrombin complex concentrate; hFVIII — human factor VIII; rFVIIa — recombinant factor VIIa; rpVIII — recombinant porcine factor VIII. Solid lines represent level of factor VIII in patients 1 (blue), 2 (orange) and 3 (green). Dotted lines represent level of inhibitor titer, which was regularly monitored throughout treatment. Treatment course of study group is presented descriptively on graph. X axis represents days of treatment. Patient 1 had longest treatment course (43 days), Patient 2 had a treatment of 26 days, and Patient 3 had shortest treatment of 20 days.

On admission, the patient's aPTT was 92 s (normal range 25.4–36.9) and FVIII level <1%. He received hemostatic treatment with a plasma-derived factor VIII concentrate. On day 7, intensification of hemorrhagic diathesis was observed. FVIII again decreased to <1% and aPTT prolonged to 84.8s. Inhibitor titer was 993 BU/mL. aPCC was administered (5,000 IU intravenously every 12 hours), which resulted in the inhibition of bleeding and an overall improvement in his clinical condition. 16 days after uneventful aPCC treatment, the dosing interval was reduced to every 24 hours. In order to facilitate the outpatient administration of coagulation factor, the patient received a peripherally inserted central catheter (PICC) due to difficulties with access. Although the patient was prepared for the procedure (7,000 IU of aPCC), his response to aPCC after the procedure was unsatisfactory: his aPTT was 122 s, and FVIII <1%. Therefore, his hemostatic treatment was switched to eptacog alpha in an infusion pump at a dosage of 90 µg/kg boluses every three hours for three days, and then every six hours for three days, twice a day for two days and once a day for 11 days. The patient was discharged from the Department of General Hematology in good general condition, with eptacog alpha 8 mg once a day for four days, then 8 mg every second day until the thoracic wound had healed. The patient's laboratory parameters and course of treatment are depicted in Figure 1.

Nurse evaluation outcomes

A large majority of surveyed personnel (75%) evaluated the infusion pump as a more comfortable and easier administration method than 4-hourly interval boluses. Only 35% claimed bolus injection treatment to be very easy, while 55% assessed infusion pump treatment as either very easy or easy. Among the greatest difficulties in bolus administration, the surveyed personnel identified frequent blood sampling (30%) and a large number of vials administered at the same time (35%), while 65% pointed to the precise time of drug administration as the greatest advantage of infusion pump administration. The greatest challenge in infusion pump treatment was considered by 65% of those surveyed to be the patient's constant connection to the infusion pump. This was also the only advantage of bolus injections named by the personnel.

Discussion

Several studies have explored the efficacy and safety of using rFVIIa administered via infusion pumps in hemophilia treatment, especially in a surgical setting [16, 18–21]. The first attempts to introduce rFVIIa infusion pump treatment came about in 1996 when Schulman et al. [18] evaluated the stability of rFVIIa in three different infusion systems. According to their study, continuous infusion with rFVIIa

was feasible and resulted in a reduction of the total dose of rFVIIa by 50–75%, depending on the behavior of the clearance. No contamination of the infusion systems was noted. However, the greatest challenge constituted thrombophlebitis at the infusion site, which was addressed by the addition of low-molecular weight heparin to an infusion bag [18]. In 2002, Stachnik et al. [20] concluded that continuous infusion of rFVIIa can enable the maintenance of constant factor concentration and thus reduce the risk of bleeding and reduce factor consumption. Over the following years, further research was conducted that came to the same conclusion [19–22]. In 2019, Négrier et al. [16] investigated the practicality and effectiveness of rFVIIa mini-pump treatment. They also conducted a survey among their nursing personnel, which revealed a preference for infusion pump administration over bolus injections [16].

Infusion pump is superior to bolus injections in terms of providing the patient with a safe and constant level of rFVIIa. Unlike the infusion pump, bolus injections require rounding the dosage of rFVIIa up or down to the nearest figure [17]. This is a crucial factor in terms of the treatment's cost-effectiveness. As long ago as 1993, Carlsson et al. [22] managed to establish an appropriate rFVIIa dose and dose interval for each patient that would allow for the maintenance of a trough factor concentration $>1\%$ at all times. They concluded that, if administered in a continuous infusion, the rFVIIa consumption would reduce to 22,000 units/year compared to 275,000 units/year with standard prophylactic dosing [22]. rFVIIa treatment is expensive: a single bolus for an 80 kg adult (a 7 mg dose) costs \$9,514 [23]. The reduction of costs offered by infusion pump treatment is not only limited to fixed dosage administration; it is also more economical in the context of nursing care. According to Pollard et al., in the United Kingdom, rFVIIa bolus dosage administration takes c.6 nursing hours per 24 hours, yet when using the pump the time amounts to only one nursing hour per 24 hours [17].

Reducing nursing time from six hours to one is also desirable because it lessens the burden on the nursing staff and lowers the risk of human error [17, 21]. The results of our questionnaire reveal infusion pump to be the preferred method of administration among 75% of the surveyed nursing personnel. Rated its greatest advantage by 65% was the possibility of administering the drug at precise time intervals, while having large numbers of vials administered at the same time (35% of surveyed) and frequent blood sampling (30%) were identified as the greatest disadvantages of bolus injections.

The concerns regarding infusion pump therapy center on the stability of the drug, the risk of infection, the risk of development of phlebitis at the infusion site, the risk of pump failure, and the possibility of inhibitor development [19]. According to SmPC, rFVIIa remains stable for 24 hours at 25 °C when stored in a 50 mL polypropylene syringe. This stability

enables infusion pump therapy [6, 14]. To date, there have not been any reports indicating early systemic infections complicating infusion pump treatment. Maintaining sterile conditions during preparation is crucial in preventing infection [19, 24–26]. Phlebitis was a common complication described in the initial studies documenting rFVIIa infusion treatment [18, 27]. Nowadays, this can easily be prevented by parallel infusion of saline [19]. The increasing quality of infusion pumps means that reports of pump failure are very rare. Frequent monitoring and thorough preparation prior to introduction of the pump treatment can be taken as preventive measures [19]. Kempton et al. [27] found no evidence of a link between constant infusion treatment and the development of inhibitors in patients with moderate or mild hemophilia who underwent intensive treatment for surgery. All reports of the development of inhibitor as a consequence of infusion pump treatment are anecdotal [19, 28, 29].

In the three patients treated in our Department, there was no compensation in hemostatic effect as a result of pump treatment. The course of management was uneventful, and no side effects were reported.

A comparison of infusion pump and bolus administration is set out in Table IV.

Conclusions

Recombinant factor VIIa via an infusion pump is more effective in maintaining hemostasis and is the preferred administration method among nursing personnel. Patients and personnel alike benefit from infusion pump administration of recombinant factor VIIa for hemophilia A.

Article information and declarations

Data availability statement

Data openly available in a public repository that issues datasets with DOIs.

Ethics statement

The work described in this article has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments involving humans; EU Directive 2010/63/EU for animal experiments; Uniform Requirements for manuscripts submitted to Biomedical journals. Our study does not require any ethics committee approval. The authors acknowledge and declare that no breach of ethical rules has been made during the preparation and publication of the study.

Authors' contributions

MW — original idea for article, patient management; MW, WR — writing of article. All authors contributed to revising and editing the manuscript.

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Conflict of interest

The authors have disclosed no conflict of interest.

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Screening of HBV infection in multi-transfused previously vaccinated patients

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Abstract

Introduction: Blood and blood products transfusion is an essential therapy for many patients. However, it carries the risk of transmission of infection. The hepatitis B virus can be transmitted by blood transfusion. This highlights the importance of combined HBsAg and anti-HBc screening to eliminate blood-transmitted HBV.

Aim of work: This study aimed to assess the prevalence of HBV among vaccinated multi-transfused patients in Egypt.

Material and methods: The study was conducted on 805 multi-transfused patients who were followed up at the outpatient hematology clinic at Cairo University Children's Hospital. Plasma blood samples were collected for an HBV rapid screening test, and serum samples were collected for serological and molecular analysis of positive cases.

Results: The results of this study results of HBV screening using RDT revealed three positive cases out of 805. The first case showed HBsAg positive, HBsAb negative, HBcAb positive, and HBeAg positive, as well as a high viral load. The second case showed HBsAg positive, HBsAb negative, HBcAb positive, HBeAg negative, and an intermediate viral load. The third case showed HBsAg negative, HBsAb positive, HBcAb positive, HBeAg negative, with an undetectable titer.

Conclusions: HBV infection persists among multi-transfused and previously vaccinated children. Therefore, the post-immunization immune status of these patients should be assessed, and strict hemovigilance should be followed.

Keywords: HBV, hepatitis B virus, chemiluminescence, PCR

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Introduction

Transfusion-related infections (TRIs) still represent a significant danger to patients worldwide. Any infection that spreads from person to person through parenteral blood or blood component delivery is referred to as a TRI [1]. Examples include Human T-cell Lymphotropic Virus (HTLV) I and II, syphilis, cytomegalovirus (CMV), malaria, hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV), hepatitis E virus (HEV), human immunodeficiency virus (HIV), and West Nile virus [2, 3].

Hepatitis B is a potentially life-threatening infection caused by HBV. The high mortality rate from liver cancer and cirrhosis makes it a severe worldwide health concern [4]. Many people are asymptomatic during the initial infection. Symptoms may appear 1–6 months post-exposure [5]. The virus is transmitted vertically through perinatal transmission or horizontally by exposure to infectious blood or bodily fluids [6]. It is recommended that all blood be tested for hepatitis B before transfusion to prevent infection. The infection can be diagnosed 30–60 days after exposure [7]. The disease was first made preventable by vaccination in

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1982 [8]. Vaccination is recommended by the World Health Organization on the first day of life if possible. Two or three more doses are required later to deliver the full effect [9]. In Egypt, mandatory vaccination against HBV was implemented in 1992 [10].

A safe and effective vaccine offers protection against hepatitis B. Preventing hepatitis B infection avoids complications, with a more than 95% success rate [11].

This study aimed to detect the prevalence of HBV infection in vaccinated and multi-transfused chronic hemolytic anemia pediatric patients attending the Cairo University Children's Hospital hematology clinic.

Patients and methods

The study was conducted on 805 multi-transfused patients with chronic hemolytic anemia who attended the hematology clinic at Cairo University from August to December 2022. These patients received the HBV vaccine as part of the Egyptian government's national vaccination program, as they were all born after 1992, the year in which the vaccine was made compulsory.

Patients' ages ranged from 2 to 24 years with a mean of 13 ± 2 SD, 51.31% were male and 48.69% female. All included patients were screened for HBsAg using an immune-chromatographic rapid diagnostic test of pharmAtos Biodiagnostic laboratories. Of the 805 patients, there were 413 (51.31%) males and 392 (48.69%) females. Cases that revealed positive screening were further subjected to the following:

- 1) **Serological study by Chemiluminescence Cobas 6000** for Hepatitis B surface Antigen (HBsAg), Hepatitis B surface Antibody (HBsAb), Hepatitis B core Antibodies (HBcAb), and Hepatitis B e Antigen (HBeAg);
- 2) **PCR** Real-time quantitative polymerase chain reaction (PCR);
- 3) **Their families were tested by Chemiluminescence Cobas 6000** for HBsAg, HBcAb.

Sample collection

3 mL of venous blood was obtained from each patient on a sterile EDTA vacutainer for follow-up in the hematology clinic, and another 3 mL of blood was collected in a plane vacutainer tube.

Material and methods

Rapid test: The rapid diagnostic test (RDT) was selected for HBV screening because of its low cost and ability for point-of-care diagnosis. The test is an *in vitro*, visually read, qualitative immunoassay for detecting HBsAg in serum, plasma, or whole blood. It is rapid and easy to use.

Interpretation of RDT results

In positive results, as the serum is added, the antigen-antibody complex migrates towards the test zone (T), where it is captured by immobilized antibodies, forming a visible line. In negative results, the antigen is absent, and there is no visible line in the (T) zone. In an invalid result, the control line zone and the test line zone do not appear within 10 minutes.

Specificity and sensitivity

A total of 1,002 serum specimens were tested with this rapid one-step HBsAg test and compared to a reference kit (**Chemiluminescence Cobas 6000**). The results showed that 16 specimens were inconsistent with this rapid one-step test and the reference kit (positive accordance rate was 99.43%, negative accordance rate was 97.25%, and total accordance rate was 98.40%). Plasma specimens were tested and compared to serum specimens. The results showed a positive accordance rate of 100%, a negative accordance rate of 98.54%, and a total accordance rate of 99.01%.

Chemiluminescence immunoassay using a Cobas 6000 system Elecsyse 601 analyzer (Roche, USA) to assess HBsAg, Anti-HBs, HBeAg.

Real-time quantitative AmpliPrep/COBAS® TaqMan® system (Roche, USA)

The AmpliPrep/COBAS® TaqMan® HBV Test, version 2.0, is an *in vitro* nucleic acid amplification test (NAT) for quantifying HBV DNA in human plasma and serum. It uses the COBAS® AmpliPrep Instrument for automated specimen processing and the COBAS® TaqMan® Analyzer or COBAS® TaqMan® 48 Analyzer for automated amplification and detection.

Results

The screening for HBsAg by immune-chromatographic rapid diagnostic test revealed 802/805 (99.63%) negative cases. Only three cases, i.e. numbers 38, 91, and 442, were positive (0.37%), as depicted in Figure 1.

The only three positive cases were serologically investigated for HBsAg, HBs Ab, HBcAb, HBeAg and the PCR quantitative assay investigated for the viral load. Descriptive data for positive cases is set out in Table I, which shows the results of the serological assessment of HBsAg, HBsAb, HBcAb, HBeAg using chemiluminescence on the three positive cases.

The results of the PCR quantitative assay for the viral load of the three cases were as follows:

- **Case 38** had a high titer of 17,000,000 IU/mL;
- **Case 91** had an intermediate titer of 20,000 IU/mL;
- **Case 442** had an undetectable titer of less than 10 IU/mL.



Figure 1. RTD results: top photo shows negative results, and other three are positive cases

Table I. Descriptive positive cases and results of serological assessment

Case number	Gender	Age	History of blood vaccination	Result of RDT	HBsAg	HBsAb	HBcAb	HBeAg	RT-PCR (IU)
Patient 38	Male	11 years	Not vaccinated	Positive	Positive	2 mIU/mL Negative	Positive	Positive	17E6
Patient 91	Female	24 years	Undocumented	Positive	Positive	2 mIU/mL Negative	Positive	Negative	2E4
Patient 442	Male	5 years	Vaccinated	Positive	Negative	20 mIU/mL Positive	Positive	Negative	Less than 10

17E6 = 17×10^6 IU; 2E4 = 2×10^4 IU; HBcAb – hepatitis B core antibodies; HBeAg – hepatitis B e Antigen; HBsAg – hepatitis B surface antigen; IU – international unit; RDT – rapid diagnostic test; RT-PCR – real-time polymerase chain reaction

An ‘undetected’ result indicates that hepatitis B virus (HBV) DNA was not detected in the serum specimen. A result of less than 10 IU/mL indicates that HBV DNA was detected. However, the HBV DNA level present cannot be quantified accurately below this lower limit of quantification of this assay.

Family members of the three cases were serologically investigated. Table II sets out the results of the HBsAg and HBcAb tests performed on them.

Case 38 was an 11-year-old boy on regular blood transfusions, and we depended on the history of vaccination given by his mother. We revealed positive HBsAg, with a low level of HBsAb (2 mIU/mL) considered negative, positive HBcAb and positive HBeAg and a high titer viral load (17,000,000 IU/mL). This patient had chronic active hepatitis B infection with high infectivity and high viral load. The low level of HBsAb reflects his low immunity to HBV infection.

Case 91 was a 24-year-old female; she was on regular blood transfusions, her vaccination history was unknown, and she had positive HBsAg, with a low level of HBsAb (2 mIU/mL) considered negative, positive HBcAb and negative HBeAg with an intermediate titer of viral load

Table II. Results of HBsAg and HBcAb of family members of three cases

Case number	No. of family members	Relation	HBsAg	HBcAb
Patient 38	3	Father	Negative	Negative
	–	Mother	Gray zone	Negative
	–	Brother	Negative	Negative
Patient 91	2	Son 1	Negative	Negative
	–	Son 2	Negative	Negative
Patient 442	5	Father	Negative	Negative
	–	Mother	Negative	Negative
	–	Sister 1	Negative	Negative
	–	Brother	Negative	Negative
	–	Sister 2	Negative	Negative

HBcAb – hepatitis B core antibodies; HBsAg – hepatitis B surface antigen

(20,000 IU/ml). This patient had chronic hepatitis B infection with low infectivity and an intermediate viral load. The low level of HBsAb reflects her low immunity to HBV infection.

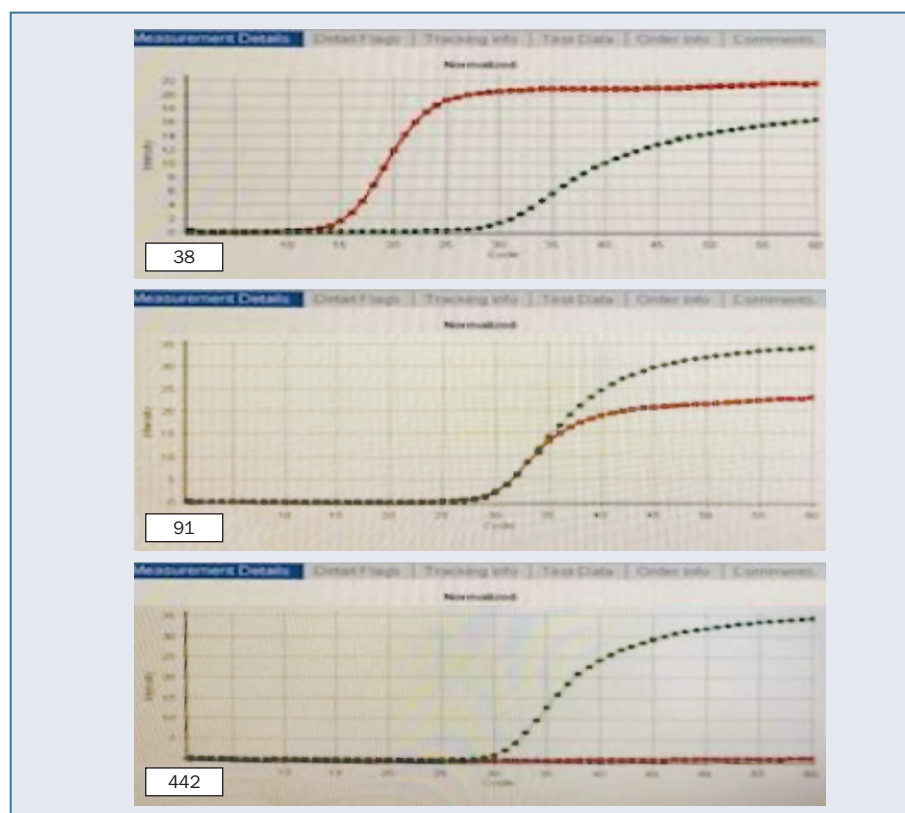


Figure 2. Polymerase chain reaction (PCR) results of cases: 38, 91, 442

Case 442 was a 5-year-old boy. He was on regular blood transfusions, and we depended on the history of vaccination given by the mother, although there was no documentation of the doses received. He had negative HBsAg, an elevated level of HBsAb (20 mIU/mL) considered positive, positive HBcAb, and negative HBeAg with an undetectable titer of HBV of less than 10 IU/mL, so the patient was diagnosed as an occult hepatitis B patient.

Family members of the three cases showed negative results for all markers (HBsAg, HBcAb) except for the mother of case 38, who showed gray zone positivity for HBsAg, and her HBsAb was negative (2 mIU/mL). PCR of this mother showed an undetectable titer of less than 10 IU as shown in Figure 2. She gave no history of blood transfusions, and her vaccination history was undocumented.

Discussion

Hepatitis B is a potentially life-threatening liver infection caused by HBV. It is a major global health problem that can cause chronic infection and put people at high risk of death from cirrhosis and liver cancer.

Hepatitis B prevalence is highest in the WHO Western Pacific Region and the WHO African Region, where 6.2% and 6.1% of the adult population are infected, respectively. In the WHO Eastern Mediterranean Region, WHO South-East

Asia Region, and WHO European Region, an estimated 3.3%, 2.0%, and 1.6% of the general population are infected, respectively. Additionally, 0.7% of people in the WHO Region of the Americas are infected [9].

According to WHO estimates, 1.5 million new cases of hepatitis B infection occur each year, and 296 million individuals worldwide were living with chronic illness in 2019. 820,000 deaths from hepatitis B are thought to have occurred in 2019, primarily from cirrhosis and hepatocellular cancer [12].

As of 2016, 27 million people (10.5% of those estimated to be living with hepatitis B) were aware of their infection, while 4.5 million (16.7%) of the people diagnosed were on treatment [13].

In 2015, Schweitzer et al. [14] reported the most robust estimates of the prevalence of chronic HBV by country and by region. Their findings highlighted both the substantial global burden of chronic HBV and the concentration of the epidemic in specific countries. This systematic review collated data from HBsAg seroprevalence studies including 109,415,627 individuals across 161 countries published between 1965 and 2013. The authors estimated that 3.61% of the global population lived with chronic HBV infection [14].

HBV prevalence by country showed significant worldwide variation, ranging from as low as 0.01% in Norway

and the UK to more than 20% in countries such as South Sudan and Kiribati [15].

In Egypt, an estimated 3.3 million people are infected with HBV, which represents a moderate endemicity. The prevalence rate declined after the universal immunization program for infants was launched in 1992 [16]. A cross-sectional analysis of the prevalence of HBV in Egypt by Ismail et al. [17] in 2017 revealed a population prevalence rate of 1.4%. This study showed marked variations in the prevalence of infection nationwide, and within-household transmission appeared to play a powerful role in sustaining HBV infection rates in Egypt [17].

HBV also spreads by needle stick injury, tattooing, piercing, and exposure to infected blood and bodily fluids such as saliva and menstrual, vaginal, and seminal fluids. Sexual transmission of hepatitis B may occur, particularly in unvaccinated men who have sex with men and in heterosexual persons with multiple sex partners or contact with sex workers [18].

Perinatal or childhood infection has been associated with few or no symptoms but has a high risk of becoming chronic. A limited number of medications can be used to treat chronic hepatitis B in these age groups effectively; however, a safe and effective vaccine is available to prevent infection [19]. Follow-up of babies born to infected (HBsAg-positive) mothers involves four doses of vaccination: the first dose at birth, then 1, 2 and 12 months later, and/or a single dose of HBIG at birth [20].

The risk factors for HBV infection have been well-characterized in the literature in general. Even so, data specific to the Egyptian context is scarce, and multiple modes of transmission have been noted [21]. Previous work in Egypt has shown strong associations between distal risk factors such as literacy and HBV risk [17].

Proximate risk factors are better characterized and include intravenous drug use, which has been strongly associated with HBV risk status in studies in Egypt, tattooing and body piercing, barbershop use, and the presence of an infected family member [17, 22]. High rates of occult HBV infection reported in some studies from Egypt reflect the higher risk of iatrogenic exposure to which they were subject [23].

One of the major concerns in transfusion medicine centers on minimizing the risk for TTIs, including HBV [24]. Despite the use of effective risk reduction measures, the goal of zero residual risk remains out of reach [25]. Since HBV vaccination was introduced into the routine immunization for children in 1992, progress has been made in improving both the scale and coordination of public efforts to tackle HBV and HCV infection through the Egyptian National Strategy and the Plan of Action for the Prevention, Care, and Treatment of Viral Hepatitis.

Researchers have suggested that the residual risk for HBV transfusion transmission is “associated with the

pre-seroconversion window period and occult HBV infection characterized by the absence of detectable HBsAg and extremely low levels of HBV DNA” [25]. Another study pointed out several factors that likely influence HBV transfusion transmission, including the volume of plasma transfused/viral load, the immune status of both recipient and donor concerning HBV, and the infecting strain of HBV [26].

Wang et al. [27] showed that HBV screening methods among blood donors differ between developed and developing countries. In developed countries like the US, blood donors are screened for HBsAg and anti-HBc. Persons positive for even one of them are disqualified based on evidence of ongoing or past infections. Combined HBsAg and anti-HBc screening virtually eliminates blood-transmitted HBV, with the rare exception of pre-HBsAg window-period donations eliminating most of the blood-transmitted HBV. In contrast, in developing countries endemic to HBV, adopting such a criterion would disqualify most volunteer blood donors. However, the advent of new viral detection technology, especially NAT, has concluded that c.10% of people with past HBV infection harbor viral DNA in their blood or blood cells [27].

The results of this HBV study screening revealed three positive cases. These initial results were further confirmed by testing the patients’ samples for various serological HBV markers using the chemiluminescence technique and quantitative viral load assay by real-time PCR.

Although the RDT is less specific than other immunological techniques (e.g. ELISA and chemiluminescence) and carries the risk of missing positive cases, this study was designed to screen as many patients as possible at the lowest cost. It is known that using more sensitive screening techniques for HBsAg (e.g. again ELISA and chemiluminescence), especially when combined with anti-HBcAb assay, can detect more positive cases with acute, chronic, or occult hepatitis B infection, or even resolved hepatitis B cases. Considering the specificity of the used assay and the results of confirmatory tests, the prevalence of HBV in the studied group was 0.37%.

It is worth mentioning that the discrepancy between positive HBsAg initial screening with RDT and negative chemiluminescence assay, in this case, comes from using another sample obtained five weeks later when the patient attended to receive another blood transfusion.

A prospective study was conducted at Mansoura University to estimate the real frequency of HBV and HCV among Egyptian β -thalassemic patients and to determine the infection-associated risk factors in this group. Fifty-eight (29.0%) were HBsAg positive, and 13 (6.5%) were anti-HBc positive by ELISA [28]. In another study conducted on transfusion-dependent thalassemic children attending the Pediatric Departments of both Sohag and Minia Universities in Upper Egypt from 2014-2015, the frequency of HBsAg positivity was 4.12% [29].

HBV screening in Cairo University Hospital's blood bank is achieved by testing the donor samples for HBsAg using ELISA following the Egyptian national standards for blood banking. However, safer screening strategies are applied worldwide.

HBsAg tests are not sensitive enough during the window period (45–50 days for most sensitive assays) for HBV infection. Likewise, in the early convalescence phase of HBV infection acute phase, as well as in chronic HBV infections, very low levels of HBsAg are often present, which are not detected by the routinely used HBsAg assays [30]. Several reports on different HBV escape mutations, in which routine screening assays did not detect HBsAg [31].

Blood-borne transmission of HBV continues to occur despite the implementation of highly sensitive screening tests for HBsAg, suggesting that these assays are still not sensitive enough to prevent all infections [25]. Unlike HBsAg, anti-HBc may be present during the chronic carrier state and at the end of an acute resolving infection where HBsAg may be undetectable. HBV-positive donors who are analyzed when they present during these stages of the disease can often be identified by either HBV NAT or by testing for anti-HBc [30]. The introduction of HBV NAT can overcome safety gaps left by HBsAg or anti-HBc testing in cases of occult HBV infection [32].

In this study, we depended on a mother's history regarding HBV vaccination, and 2/3 positive cases had a low HBsAb titer. The Egyptian Ministry of Health has adopted a schedule for HBV immunization that involves three doses of yeast-recombinant hepatitis B vaccine administered to all infants at 2, 4, and 6 months, to coincide with other compulsory vaccines.

Optimal immunization is critical for all patients with thalassemia, especially transfused patients. Patients need to be immunized against hepatitis B, especially patients on chronic transfusions. Annual monitoring of titers and booster immunizations, when indicated, will ensure patients are well protected [33].

Salama et al. [34] conducted a study to assess the effectiveness of the HBV vaccination program among fully vaccinated children in Egypt. A total of 3,600 children aged from 9 months to 16 years who were fully vaccinated with the HBV vaccine during infancy were recruited. Sera were evaluated for HBsAg, anti-HBcAb (total), and quantitative detection of hepatitis B surface antibody by ELISA. Samples positive for HBsAg or anti-HBcAb were subjected to quantitative HBV PCR. Sero-protection was detected among 2,059 children (57.2%) with mean titers of 75.4 ± 3.6 IU/L compared to 3.1 ± 2.1 IU/L among non-seroprotected children. HBsAg was positive in 0.11%, and breakthrough infection was found in 0.36% and 0.39%, depending on the positivity of anti-HBc and DNA detection, respectively. The prevalence of HBV infection was significantly higher among children aged ≥ 7 years (0.59%) compared to 0.07%

among those aged under seven [34]. In a cross-sectional study by El-Beshlawy et al. [35], 100 multi-transfused children were included, and screening for HBsAg, HBcAb by ELISA, only 12% of patients were either acutely or chronically infected with HBV. 46% were immune due to previous vaccination, and 39% were not protected from HBV infection. However, complete HBV vaccination in this study was achieved in 78/100 patients. The authors also found that among all multi-transfused children in the study, one in three of those who had completed their vaccinations were not protected [35].

El-Beshlawy et al. [35] showed HBsAg positivity had been 16.48% before the implementation of the national HBV vaccination. Establishing a vaccination program against HBV has had a notable impact on the prevalence of hepatitis B infection [16].

This decrease in the prevalence of hepatitis B infection reflects the efficacy of the vaccination program, and the effective screening of blood and blood products [36]. Despite the decline in the prevalence of HBsAg among Egyptian multi-transfused children over recent decades [37], it is still high compared to other countries, and this could be attributable to the high infection rates among those children despite vaccination against HBV, as they might not have responded adequately to vaccination or may not be fully vaccinated [34]. Post-vaccination infections may be a result of viruses with surface (S)-gene mutations in a region critical for antibody reactivity to the hepatitis B surface antigen (anti-HBs) [38].

Although blood is screened for HBV in blood banks in Egypt, transfusion-related HBV transmission may occur because screening in most blood banks is performed only for HBsAg, not for core antibodies [39, 40]. This has led to calls for the implementation of the anti-HBc test as a routine assay, which would undoubtedly eliminate possible HBV-infected blood units. Rejection of these units would decrease the risk of HBV transmission, particularly in immune-compromised recipients.

The results of this study together with those of Salama et al. [34] and El-Faramawy et al. [39], still reveal a gap regarding vaccination schedules that requires additional effort to ensure that the entire population has strictly completed their vaccinations, particularly in high-risk groups of patients.

In this study, the family members of the three cases were serologically investigated for HBsAg and HBcAb, and they all showed negative results except for the mother of case 38, who showed gray zone positivity for HBsAg while her HBsAb was negative (2 mIU/mL). Real-time PCR on her sample showed a titer of less than 10 IU. She gave no history of blood transfusions, and her vaccination history was unknown. Follow-up of this mother is needed, as she might have had a recent infection. Although the transmission mode is not yet apparent, the role played by household

contact in active infections has been highlighted in a study by Talaat et al. [23].

In conclusion, to reduce the incidence of HBV in multi-transfused patients, we recommend active immunization against HBV for all patients on repeated blood transfusion therapy, plus strict follow-up and hemovigilance.

Article information and declarations

Data availability statement

Data that supports the findings of this study is available on request from the corresponding author.

Ethics statement

Informed consent from all individuals enrolled in the study was obtained.

Authors' contributions

AA — idea for study, study design; NE — study design, manuscript revision; ND — practical part of screening, manuscript preparation; FAWAM — manuscript preparation; IY — clinical enrollment and follow-up of cases, manuscript revision.

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Conflict of interest

The authors declare no conflict of interest

Supplementary material

None.

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Endothelial dysfunction and thrombosis in polycythemia vera

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Abstract

Polycythemia vera (PV) is a chronic myeloproliferative neoplasm with an increased risk of thrombotic events. Endothelial dysfunction is a pathogenetic mechanism contributing to thrombus formation in PV. The presence of the *JAK2 V617F* mutation is associated with an increased risk of thrombosis due to changes in endothelial homeostasis mediated by overexpression of pro-adhesive and proinflammatory agents. Cytoreductive treatment that decreases the *JAK2* allele burden and inhibits the *JAK/STAT* signaling pathway is potentially more effective in thrombosis prevention than drugs that are less effective in the reduction of the *JAK2* allele burden.

This review aims to present the spectrum of endothelial dysfunction and the impact of cytoreductive treatment on the condition of endothelial cells and thrombosis risk in patients suffering from polycythemia vera.

Keywords: polycythemia vera, endothelium, endothelial dysfunction, thrombosis, thrombotic complications

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Introduction

Polycythemia vera (PV) is a chronic myeloproliferative neoplasm characterized by increased red blood cell mass and blood viscosity. Thrombotic events are observed in c.40% of patients, and are the leading cause of morbidity and mortality in PV [1, 2]. Arterial thrombosis comprises 60–70% of all thromboembolic events in PV [3]. Of these, the most frequent are cerebrovascular events (stroke and transient ischemic attack), which constitute 25.2% and 24.5% of all arterial events, respectively [4]. Arterial occlusion less frequently involves coronary arteries and peripheral arteries [3, 4]. Venous thrombosis includes pulmonary embolism, deep vein thrombosis, cerebral vein thrombosis, and splanchnic venous thrombosis *i.e.* thrombosis located in the portal vein, splenic vein, porto-mesenteric vein, or hepatic vein (Budd-Chiari syndrome) [5, 6]. Venous events

in PV often occur in atypical locations, most frequently in splanchnic veins, which constitute 45% of all venous events [7]. Thrombosis may be the first manifestation of PV, which is observed in 12–25% of patients [1, 7, 8]. In a group of patients under the age of 25 diagnosed with PV and treated with cytoreduction, the reason for treatment implementation in 24.6% of cases was thrombotic events [9]. Highest rates of thrombosis in PV usually occur before, or at the time of, diagnosis, and decrease over time. This is probably due to the effects of cytoreductive treatment (64% of thrombotic events at the time of diagnosis or before vs. 36% during follow-up) [1]. However, an increased risk of developing thrombotic events persists throughout the patient's lifetime [10]. The rate of thromboembolic events in PV after diagnosis is c.2.6% per year [11, 12]. Thrombosis after diagnosis occurs in 22% of PV patients [7], and 16% of PV patients develop at least one thrombotic event after

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treatment initiation, which occurs more frequently in high-risk PV patients compared to low-risk [13]. The 20-year rate of thrombotic events in PV is 26%. Contrastingly, the 20-year rates of two other life-shortening complications, *i.e.* fibrotic transformation and leukemic transformation, are 16% and 4%, respectively [14]. Thus, the prevention of thrombotic complications is one of the main treatment goals in PV.

The pathogenesis of thrombosis in PV is complex and multifactorial, and involves both blood and vascular cells. Increased risk of thrombosis is associated with elevated blood cell count, *JAK2 V617F* allele burden, and activation of platelets, leukocytes, and endothelial cells [15, 16]. Tumor-mutated clone and circulating malignant cells induce an inflammatory host response which leads to generalized inflammation and promotes a prothrombotic state in PV. Malignant cells produce cytokines responsible for the prothrombotic phenotype of vascular endothelial cells [17].

Endothelial dysfunction is one of the factors associated with a high propensity for thrombosis in PV. Physiologically, the endothelium participates in the maintenance of vascular integrity and generates an antithrombotic surface. The dysfunction of endothelial cells observed in PV causes a series of changes that create pro-adhesive and prothrombotic blood conditions represented by an activation and adhesion of platelets, leukocytes, and erythrocytes to the endothelium, the formation of circulating platelet-leukocyte aggregates, neutrophil extracellular traps and microparticles, the generation of thrombin and the release of pro-inflammatory cytokines [5, 18, 19].

The prothrombotic phenotype of endothelial cells with the *JAK2* mutation is reflected in the overexpression and increased secretion of inflammatory, pro-adhesive, and endothelial cell activating factors: von Willebrand factor, P-selectin, E-selectin, soluble thrombomodulin, vascular cell adhesion protein 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1), endothelial leukocyte adhesion molecule 1 (ELAM-1), vascular endothelial growth factor (VEGF), IL-6 and IL-33 receptor [20–25].

The molecular basis of endothelial dysfunction in PV includes a *JAK2 V617F* expression promoting thrombosis by inducing endothelial P-selectin expression, which may be reversed by cytoreductive therapy [23]. Molecules that participate in the adhesion of erythrocytes to the endothelium in PV are the Lutheran/basal cell adhesion receptor (Lu/BCAM) phosphorylated by the *JAK2 V617F* mutation and the endothelial alpha-5-laminin chain [26]. *JAK2* mutation expression in the endothelium causes tight adhesion of leukocytes to endothelial cells [21]. *JAK2* is also involved in collagen-induced platelet activation [27]. Inhibition of *JAK/STAT* signaling limits the overexpression of several genes that are involved in venous stenosis and thrombosis due to pro-inflammatory and pro-adhesive properties [22]. Inhibition of *JAK/STAT* signaling prevents thrombogenesis in PV.

Observational studies have confirmed the involvement of the *JAK2 V617F* mutation in the increased risk of thrombosis. *JAK2* variant allele frequency (VAF) >50% correlates with the risk of venous thrombotic events. This is an important risk factor for venous thrombosis, and also in conventionally low-risk patients [28].

Therefore, a reduction in the *JAK2* mutant allele burden seems to be a priority in the treatment of PV patients. The second, and equally important, goal of modern PV therapy should be to extinguish chronic inflammation.

Effect of treatment on endothelial function in PV

Thrombosis history and age over 60 are the two crucial criteria for the stratification of risk in patients and the choice of treatment regimen [29]. Cytoreductive treatment is indicated in patients with PV conventionally defined as high-risk, and in some specific situations in the low-risk patient group, such as inadequate hematocrit (Hct) control with phlebotomies (*i.e.* a need for 6+ phlebotomies per year), symptomatic splenomegaly, severe pruritus, persistence of constitutional symptoms, leukocytosis $> 15 \times 10^9/L$, thrombocytosis $\geq 1,500 \times 10^9/L$, and relevant cardiovascular risk (hypertension, ischemic heart disease, diabetes mellitus) [29, 30].

The three main goals of PV treatment are the prevention of thrombotic complications, improving quality of life by releasing or alleviating constitutional symptoms, and inhibiting the progression of fibrosis in the bone marrow. Good disease control is reflected in the maintenance of Hct below 45%. PV patients with Hct <45% have a significantly lower rate of cardiovascular death and of major thrombotic events [31]. Historically, the primary method of maintaining target Hct values has been phlebotomy. But nowadays it is known that phlebotomies alone do not decrease the risk of PV progression to the fibrotic phase; therefore, they are mainly used as adjunctive therapy in order to rapidly lower Hct in high-risk patients or periodically in low-risk patients [29, 32]. In the large cohort of PV patients included in the European Collaboration on Low-dose Aspirin in Polycythemia Vera (ECLAP) study, the maintenance of Hct <45% at 12 months was achieved in more than half of patients treated with HU but in only 31% of patients treated with phlebotomy alone. Phlebotomy was also less effective in controlling leukocytosis. The advantages of HU over phlebotomy were seen in reduced overall mortality, cardiovascular events, and myelofibrosis transformation [4].

The literature shows that the number of phlebotomies per year correlates with an increased risk of thrombosis in PV. Patients treated with HU who also needed at least three phlebotomies per year to maintain Hct control have been shown to have a significantly higher rate of thrombosis compared to patients who required fewer venesections

(20.5% vs. 5.3% at 3-year follow up). The need for phlebotomy identifies a subgroup of PV patients with excessive cell proliferation and an increased risk of thrombosis [33]. A clinical correlation between the need for phlebotomy and the risk of thrombotic events has also been shown in the MAJIC-PV trial. The time to the first thrombotic event within the first three years was associated with the average number of venesections [34]. Phlebotomy-induced iron deficiency may be responsible for an increased risk of thrombotic events [35–37]. Iron deficiency leads to reactive thrombocytosis by increasing the expression of vascular endothelial growth factor A (VEGFA) and affecting megakaryocyte differentiation [38]. Jimenez et al. [37] analyzed a link between iron deficiency and thrombotic events in an animal model. They observed a larger diameter of arterial and venous thromboses and a faster growth of thromboses in a rat model of iron deficiency. Platelet count correlated with the final thrombus dimension. Furthermore, the plasma and platelet expression of P-selectin was higher in the iron deficiency sample. All the above changes were reversible, and normalized after iron replacement therapy [37]. Iron deficiency leads to the activation of hypoxia-inducible factors (HIFs), which results in overexpression of several prothrombotic genes [36]. Among PV patients, those with iron deficiency had higher levels of mRNA detected in granulocytes and platelets of thrombospondin 1 (*THBS1*), P-selectin (*SELP*), interleukin 1 receptor-associated kinase 1 (*IRAK1*), and plasminogen activator inhibitor-1 (*SERPINE1*). Increased expression of prothrombotic genes mediated by iron deficiency has been observed in PV, essential thrombocythemia, and Chuvash polycythemia [36].

The cytoreductive treatment for PV includes hydroxycarbamide (HU), interferon- α (IFN- α), and ruxolitinib.

Hydroxyurea

Despite the development of new drugs *i.e.* pegylated interferon alpha 2a (PEG-IFN- α), ropeg interferon alpha 2b (ropeg-IFN- α), and ruxolitinib, HU is still considered to be among the front-line therapies in high-risk PV. HU is an inhibitor of ribonucleotide reductase, the enzyme responsible for the conversion of ribonucleotides to deoxyribonucleotides. HU inhibits DNA synthesis in the S phase of the cell cycle [39].

The effect of HU on the vascular endothelium has been investigated primarily in sickle cell anemia, where HU is the only form of pharmacotherapy dedicated to this disease, which decreases the frequency and severity of vaso-occlusive crises. HU significantly reduces endothelial release of the vasoconstrictor peptide endothelin-1 (ET-1) by down-regulating ET-1 gene expression. This effect is reversible and dose-dependent [40]. HU also reduces the expression of VCAM-1 in endothelial cells [40].

However, a negative effect of HU on the endothelium has also been observed, potentially promoting thrombosis.

HU has been shown to increase the synthesis of ICAM-1, which is typically expressed in endothelial cells and cells of the immune system [40]. In an analysis of sickle cell anemia patients, those treated with HU had a significantly lower level of thrombospondin-1 than those not treated with HU [40]. Thrombospondin-1 is an adhesive glycoprotein that mediates the adhesion of red blood cells and platelets to the vascular endothelium. However, the levels of a disintegrin-like metalloproteinase with thrombospondin motif type 1 member 13 activity (ADAMTS13:Act) were shown to be significantly higher in patients receiving HU, indicating that HU acts to reduce thrombus formation [41]. HU increases nitric oxide (NO) production in endothelial cells through endothelial nitric oxide synthase (eNOS) phosphorylation [42]. Under laboratory conditions, a prominent elevation in HU-induced NO levels has been observed in a human bone marrow endothelial cell line [42]. In patients with sickle cell anemia, HU treatment has been associated with a decrease in the level of the thrombin-antithrombin complex and a decrease in the plasma level of the soluble vascular cell adhesion molecule-1 (sVCAM-1), which reflects a lower prothrombotic activity [43].

Observations on HU activity in sickle cell anemia studies suggest that HU may also prevent PV-related thrombotic complications due to influencing the activity of the endothelium by decreasing the expression of cell adhesion molecules, and increasing NO synthesis. However, Brusson et al. [44] showed adverse effects of HU on red blood cells and endothelial cell physiology in PV that may contribute to thrombus formation. In contrast to IFN- α , HU improves the adhesion of erythrocytes to endothelial laminin through increased Lu/BCAM phosphorylation [44].

Interferon

IFN- α is another cytoreductive treatment option for patients with PV. Currently, recombinant forms of IFN- α are available (rIFN- α) and are used in PV therapy: PEG-IFN- α and ropeg-IFN- α . The development of pegylated forms of IFN- α has allowed for less frequent administration, and has increased tolerance and efficacy [45]. Ropeg-IFN- α , in contrast to PEG-IFN- α , is a monopegylated rIFN- α , which extends elimination half-life, resulting in less frequent dosing and better tolerability [46]. The mechanism of action of rIFN- α in PV includes downstream activation of JAK/STAT signaling by binding to the interferon-alpha/beta receptor (IFNAR). Down-regulation of JAK/STAT signaling leads to a series of antiproliferative, antiangiogenic, pro-apoptotic, and immunomodulatory effects affecting virtually all immune cells, especially T cells, macrophages, and natural killer cells [47, 48].

rIFN- α not only normalizes elevated blood cell counts in patients with PV, but also can decrease the mutant *JAK2 V617F* allele burden [49–51]. After long-term treatment,

rIFN- α may even induce minimal residual disease, with *JAK2* V617F VAF <1% and complete histological normalization of the bone marrow in some patients [45, 52–54]. Data from clinical trials that confirm the efficacy and safety of rIFN- α , and their ability to modulate the natural history of PV, supports the use of rIFN- α as a first-line treatment option for PV, especially for younger patients with indications for cytoreduction [32, 55, 56].

The effect of rIFN- α on the endothelium has been the subject of research in numerous diseases, including systemic lupus erythematosus (SLE). The literature shows the negative impact of type I interferon on the endothelial function in SLE [57, 58]. A link between type I interferons and decreased eNOS expression and NO bioavailability has been observed [59]. Faillie et al. analyzed the impact of rIFN- α and HU on the biological hemostatic profile of patients with myeloproliferative neoplasms. They noted that rIFN- α reversibly increased endothelial activation markers [60]. In patients treated with rIFN- α , factor VIII activity, von Willebrand factor antigen concentration, and von Willebrand factor activity were all significantly higher, while protein S activity was significantly lower compared to patients treated with HU and untreated patients. The fibrinogen concentration was higher in rIFN- α -treated patients than in untreated patients, but not in HU-treated patients. After discontinuation of rIFN- α therapy, the altered hemostatic parameters were restored to normal levels: the von Willebrand factor antigen concentration decreased from 182% to 92%, the von Willebrand factor activity decreased from 172% to 92%, the factor VIII activity decreased from 158% to 112%, the fibrinogen concentration decreased from 3.7 to 3.1 g/L, and the protein S activity increased from 62% to 87% [60]. Despite the reversible changes in the biological hemostatic profile of the endothelium, rIFN- α therapy reduces the risk of thrombosis in PV.

The mechanisms responsible for thrombotic risk reduction include the inhibition of malignant cell proliferation, the stimulation of apoptosis, and increased immunogenicity against malignant clone cells [61]. rIFN- α therapy leads to the achievement of complete hematological response and a reduced *JAK2*V617F allele burden. The antithrombotic effect of rIFN- α influences two primary mechanisms of the prothrombotic tendency in PV: excessive circulating malignant cells, and chronic inflammation [62]. rIFN- α at the molecular level prevents thrombotic events. rIFN- α inhibits the generation of reactive oxygen species (ROS) in myeloproliferative neoplasms by downregulating several oxidative stress genes and upregulating antioxidative defense genes [63]. Both the *JAK2* mutation and ROS are involved in thrombosis formation [64, 65]. rIFN- α reduces genotoxic damage to hematopoietic stem cells, decreasing the risk of the appearance of additional mutations, which promote clonal evolution and disease progression to myelofibrosis and leukemic transformation [63].

The outcomes of a large single-center retrospective study supported the use of rIFN- α rather than HU and phlebotomy in the treatment of both high-risk and low-risk patients with PV. In high-risk patients, the 20-year myelofibrosis-free survivals for rIFN- α , HU, and phlebotomy were 89%, 41%, and 36%, respectively, and 20-year overall survivals were 66%, 40%, and 14%, respectively. In low-risk patients, the analogic parameters were as follows: 84%, 65%, and 55% of the patients achieved 20-year survival without progression to myelofibrosis. Overall survivals were 100%, 85%, and 80%, respectively [32]. A randomized controlled trial comparing the efficacy and safety of PEG-IFN- α and HU found no significant difference between these agents in the rates of thrombotic events, although the follow-up period (12 months) was not long [66]. Final results from the 6 $\pm$ year follow-up studies PROUD-PV and its extension CONTINUATION-PV comparing ropeg-IFN- α and HU/best available treatment (BAT) showed that the probability of event-free survival was significantly higher in the ropeg-IFN- α arm compared to the HU/BAT arm (0.94 vs. 0.82, $p = 0.04$). Adverse events included thromboembolic events, progression to myelofibrosis, progression to acute leukemia, and death [56].

Ruxolitinib

Ruxolitinib is a Janus-activated kinase (JAK) inhibitor that selectively inhibits JAK1 and JAK2. Inhibition of JAK kinase results in interruption of the cytokine and growth factor signaling pathway (*JAK/STAT*), leading to a decrease in the level of pro-inflammatory cytokines and chemokines that counteract inflammatory reactions [67].

Ruxolitinib is indicated in high-risk PV patients who are resistant or intolerant to HU, especially with symptomatic splenomegaly or severe drug-resistant pruritus, and in patients with symptoms typical of post-PV myelofibrosis [29]. The benefits of ruxolitinib therapy have been confirmed in clinical trials: improving overall survival and event-free survival, and decreasing the allele burden of *JAK2* V617F [34]. Ruxolitinib due to *JAK/STAT* inhibition has the potential to reduce endothelial prothrombotic activation and proadhesive interactions between endothelial cells and leukocytes. *JAK/STAT* inhibition reduces endothelial cell activation mediated by TNF- α , and decreases the secretion of endothelial proadhesive and procoagulant factors *i.e.* P-selectin, von Willebrand factor, IL-6, endothelial tissue factor, and urokinase plasminogen activator [68]. Analysis of the endothelialized model demonstrated a significant increase in leukocyte and platelet velocity and a reduction in cell adhesion under the influence of ruxolitinib [69]. Ruxolitinib limits the formation of extracellular neutrophil traps [70] and reduces the expression of von Willebrand factor, VCAM-1, and P-selectin [69]. However, strong evidence in favor of ruxolitinib for the prevention of cardiovascular events is still lacking [71]. Ruxolitinib is more effective than BAT

in achieving a molecular response, which correlates with a reduced risk of thrombosis [71]. In the MAJIC study, patients with a sustained complete or partial molecular response had no thrombotic complications during follow-up, compared to a thrombotic event rate of 19.1% in patients without at least a partial molecular response [72]. A meta-analysis of four randomized controlled trials: RESPONSE, RESPONSE-2, MAJIC, and RELIEF, did not show a clear advantage of ruxolitinib in preventing thrombosis. The ability of ruxolitinib to reduce the risk of thrombosis was suggested (thrombosis ratio 3.09% and 5.51% of patients per year, for ruxolitinib and BAT, respectively), but it was not statistically significant ($p = 0.098$) [71].

Discussion

Endothelial dysfunction is one of the main mechanisms that contribute to thrombosis in PV. The *JAK2 V617F* mutation does not only affect hematopoietic stem cells and multipotent progenitor cells, but is also present in the endothelial cells of patients with myeloproliferative neoplasms and Budd-Chiari syndrome [73–75]. This mutation changes the phenotype and functions of endothelial cells. It induces the overregulation of several genes and pathways involved in inflammatory processes and cellular adhesion, with consequences in terms of thrombosis formation [22, 23, 59].

Historically, phlebotomies were the basis of PV treatment. It must be emphasized that phlebotomies do not improve patient quality of life. PV patients treated with phlebotomies have been shown to have significantly more severe fatigue, abdominal discomfort, inactivity, problems with concentration, night sweats, pruritus, and bone pain [76]. Phlebotomy alone was insufficient to keep Hct at a safe level in the ECLAP study, which showed the advantage of HU over phlebotomy in the Hct control at all time points analyzed *i.e.* after 12, 24, and 36 months [4]. Moreover, recent studies have warned against the excessive use of phlebotomies to control Hct in PV, suggesting that phlebotomies may contribute to thrombosis by inducing the overexpression of prothrombotic genes mediated by iron deficiency [35–37].

Interesting conclusions were drawn from a study by Faille et al. [60], which showed that IFN- α changes endothelial homeostasis to a more prothrombotic one. In general, their study showed that IFN- α -induced elevation of prothrombotic biomarkers may be considered a biological side effect of this treatment [60]. The clinical significance of the above observations, which may reflect an increased susceptibility to thrombotic events, needs to be further investigated since it does not translate into a clinically increased incidence of thrombotic complications in patients treated with rIFN- α . However, particular attention may need to be paid to patients treated with IFN- α who have additional risk factors for thrombotic events. Data from observational studies and randomized controlled

trials clearly shows the benefit of rIFN- α over HU in the treatment of patients with PV in terms of longer event-free survival, including thrombotic events [32, 50, 55, 56]. These studies support the use of rIFN- α in the early treatment of PV to reduce thrombotic complications, prevent fibrotic transformation, and prolong survival.

JAK2 kinase inhibitors, unlike HU, down-regulate *JAK/STAT* signaling pathway and have the greatest potential to reduce the risk of thrombosis. Given this, it is surprising that data from post-marketing reports of JAK2 kinase inhibitors efficacy shows that this drug class can increase the incidence of pulmonary thrombosis and that the use of ruxolitinib may be risky in patients with portal vein thrombosis [77]. However, randomized controlled trials do not support this observation. A meta-analysis of randomized controlled trials suggest the superiority of ruxolitinib over alternative therapies in reducing the rate of thrombotic complications (3.09% and 5.51% of patients per year, respectively), although these results were not statistically significant [71].

Conclusions

Endothelial dysfunction in PV plays a key role in the pathogenesis of thrombosis and is strongly associated with the presence of the *JAK2* mutation. *JAK/STAT* signaling promotes the overexpression of inflammatory and pro-adhesive factors. Therefore, drugs that inhibit *JAK/STAT* signaling are more effective in preventing thrombosis in patients with PV. To date, no studies have been conducted directly comparing the effectiveness of rIFN- α to that of ruxolitinib in patients with PV, also in terms of reducing the tendency to thrombotic complications. The results of such studies would be very interesting, and would allow for a better determination of the role of both these drugs in the therapy of PV.

Article information and declarations

Authors' contributions

WML — idea for article, analysis and interpretation of data, drafting article, critical revision; PK — idea for article, analysis and interpretation of data, drafting article; PL — idea for article, analysis and interpretation of data, drafting article; TS — idea for article, critical revision, final approval of version to be published.

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Conflict of interest

The authors declare no conflict of interest.

Supplementary material

None.

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Checkpoint inhibitors as potential therapeutics in acute myeloid leukemia

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Abstract

Despite recent progress in treatment methods, acute myeloid leukemia (AML) continues to pose significant clinical challenges and is associated with generally unfavorable prognoses. Patients considered fit for intensive therapy are usually treated with cytarabine-anthracycline-based induction chemotherapy. Allogeneic hematopoietic stem cell transplantation (allo-HSCT) is recommended for patients with adverse-risk AML and most patients with intermediate-risk AML. The standard therapy for patients who are deemed too poorly for intensive treatment is the combination of azacitidine and venetoclax. AML cells can escape the immune system through various mechanisms, including reduced expression of MHC complex molecules, ligand shedding, manipulation of chemical signaling, and enhanced inhibitory ligand expression. Increased expression of ligands for T-cell-regulation checkpoints is present in AML cells and correlates with worse outcomes. Therefore, this article reviews the current research progress in immune checkpoint inhibitors in AML.

Keywords: checkpoint inhibitors, therapy, acute myeloid leukemia, survival

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Introduction

Despite recent progress in treatment methods, acute myeloid leukemia (AML) continues to pose significant clinical challenges and is associated with generally unfavorable prognoses, with an estimated 5-year overall survival (OS) rate of c.30% that varies between different age groups [1].

Prognosis estimation in AML involves a multi-faceted assessment that considers various clinical and biological aspects, including patient characteristics, disease-related factors, and disease ontogeny. Based on the patient's disease history, AML can be classified into two groups: *de novo* AML and secondary AML. Secondary AML that arises from a pre-existing hematological disease or following treatment with chemotherapy or radiation accounts for 15–30% of all cases, and has a generally worse prognosis

compared to primary AML [2]. Biological factors such as cytogenetic abnormalities, gene mutations, and chromosomal aberrations are the basis for the European Leukemia Net (ELN) criteria that stratify AML into categories of favorable, intermediate, or adverse risk. The ELN criteria are dynamic and were last updated in 2022 due to novel molecular findings. Risk stratification strongly influences treatment decisions [3].

Currently, the treatment regimen for young, transplant-eligible patients with AML who are considered to be fit for intensive treatment consists of two phases: complete remission (CR) induction and a consolidation phase. CR induction usually involves cytarabine-anthracycline-based induction chemotherapy, commonly following the '7+3' regimen [4]. If the leukemia fails to respond sufficiently to the initial induction therapy, a second induction is started.

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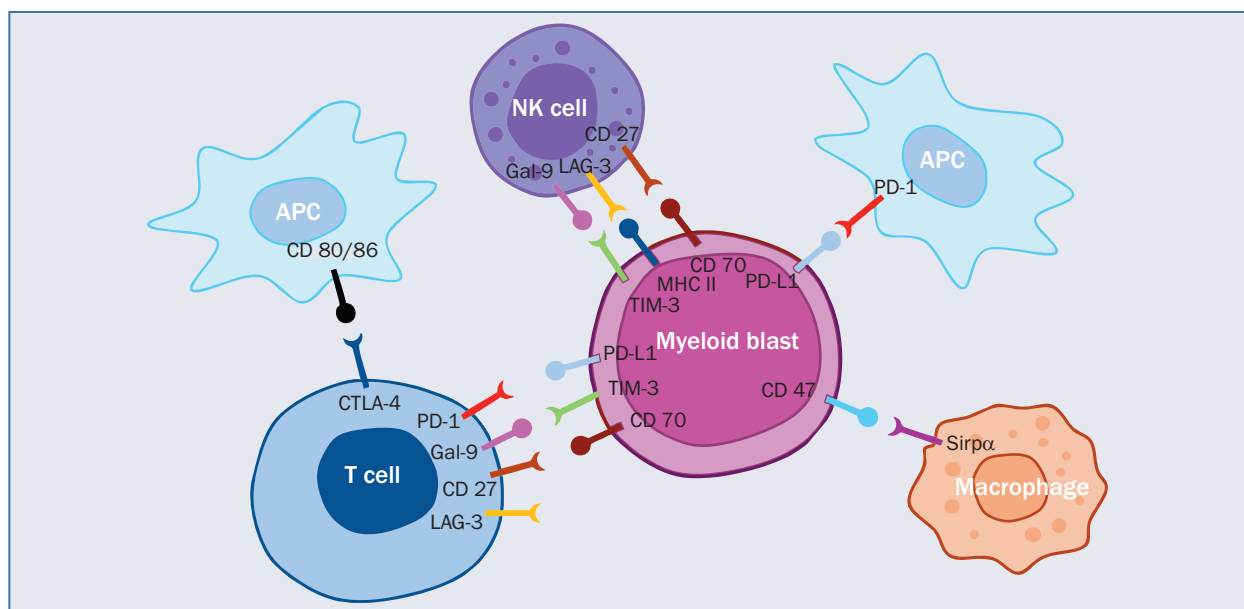


Figure 1. Checkpoint inhibitors and ligands in acute myeloid leukemia; APC — antigen-presenting cell; CTLA-4 — cytotoxic T-lymphocyte associated protein 4; Gal-9 — galectin-9; LAG-3 — lymphocyte-activation gene 3; MHC II — class II major histocompatibility complex; PD-1 — programmed cell death protein 1; PD-L1 — programmed cell death ligand 1; SIRPα — signal regulatory protein alpha; TIM-3 — T-cell immunoglobulin and mucin domain-3

Daunorubicin + AraC + cladribine (DAC) and cladribine + AraC + mitoxantrone (CLAM) are equally effective [5].

Research by the Polish Adult Leukemia Group has shown the beneficial effect of adding cladribine to the '7+3' regimen in newly diagnosed AML patients, including those under 60 years old [6] as well as those over 60 [7]. In elderly patients unfit for intensive chemotherapy, the combination of cladribine with low-dose cytarabine has been proved to be a more effective therapeutic option compared to low-dose cytarabine alone [8]. Additionally, gemtuzumab ozogamicin can be used in combination therapy with daunorubicin and cytarabine for the treatment of patients aged 15 years and older with previously untreated *de novo* CD33 positive AML, except acute promyelocytic leukemia [9].

For patients with a therapy-related AML (t-AML), or AML with myelodysplasia-related changes (AML-MRC), CPX-351, a liposomal formulation of daunorubicin and cytarabine, is an alternative to the classical regimen. The use of targeted therapy is standard for leukemia with specific mutations. The IDH1 and IDH2 inhibitors ivosidenib and enasidenib are approved for patients with AML with mutations of the respective genes [10]. Midostaurin — a type I first-generation FMS-like tyrosine kinase 3 (FLT3) inhibitor — is approved in combination with standard induction and consolidation chemotherapy for FLT3 mutation-positive patients. Gilteritinib — a second-generation FLT3 inhibitor — can be used for relapsed/refractory AML in the same group of patients [11, 12].

Lower-intensity therapy with azacitidine and venetoclax for patients who do not tolerate intensive remission induction therapy might prolong survival, but only rarely results in long-term disease control [13].

Recurrence of AML is the most significant negative prognostic factor, occurring in c.30% of pediatric patients and reducing the 5-year OS to below 30% [14]. In the adult population, relapse is the most common cause of treatment failure, with a 5-year OS rate of c.10% for patients with disease recurrence [15].

To prevent relapse, post-remission consolidation therapy with high-dose cytarabine (HiDAC) is commonly used to eliminate residual, undetectable disease, although the optimal number of cycles and the timing remain under evaluation [16].

Allo-HSCT remains the most effective option due to its graft-versus-leukemia (GVL) effect. However, in many cases, the toxicity might outweigh the benefit [17], and in 30–70% of patients, allo-HSCT fails due to disease relapse, which significantly lowers the survival rate [18]. Therefore, the therapy is recommended for patients with adverse-risk AML and for most patients with intermediate-risk AML.

The curative potential of allo-HSCT is one of the reasons behind the growing interest in the potential role of immunotherapies in treating myeloid neoplasms, including AML.

AML cells can escape the immune system through various mechanisms, including reduced expression of MHC complex molecules, ligand shedding, manipulation of chemical signaling, and enhanced inhibitory ligand expression [19, 20].

Table I. Overview of discussed immune checkpoints, their physiological function, and corresponding immune checkpoint inhibitors (ICIs)

Immune checkpoint	Physiological function	ICIs
CTLA-4	Inhibition of T-cell activation through competition with CD28 for binding to CD80/86 ligands	Ipilimumab
PD-1/PD-L1 axis	Inhibition of T-cell activation, proliferation and cytokine production	Nivolumab, pembrolizumab, durvalumab, avelumab, atezolizumab
TIM-3/Galectin-9 axis	Promotion of T-cell exhaustion; enhancement of treg cells	Sabatolimab
LAG-3	Reduction of T-cell proliferation and cytokine production upon binding to MHC class II and other ligands	Relatlimab
CD27/CD70 axis	Promotion of T-cell activation and proliferation in healthy individuals or immunosuppressive effect resulting from tumor-associated CD70 overexpression and chronic stimulation of axis	Cusatuzumab
CD47/SIRPα axis	Prevention of phagocytosis of healthy cells	Magrolimab, evorpacept, lemozaparlumab

CD27/CD70 – cluster of differentiation 27/cluster of differentiation 70; CD47 – cluster of differentiation 47; CTLA-4 – cytotoxic T-lymphocyte associated protein 4; LAG-3 – lymphocyte-activation gene 3; PD-1 – programmed cell death protein 1; PD-L1 – programmed cell death ligand 1; SIRPα – signal regulatory protein alpha; TIM-3 – T-cell immunoglobulin and mucin domain-3

Increased expression of ligands for T-cell-regulation checkpoints, including cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), B7-H3, and T-cell immunoglobulin and mucin-domain containing-3 (TIM-3), is present in AML cells and correlates with worse outcomes [21].

Implementing immune checkpoint inhibitors (ICIs) has been successful in many solid tumors, but the same cannot be said about myeloid malignancies. The main reasons behind the limited progress in this area are likely a lower mutational burden and higher heterogeneity than solid tumors [19, 22]. This review will present the current research progress in immune checkpoint inhibitors in AML.

CTLA-4 blockade

Cytotoxic T-lymphocyte antigen-4 (CTLA-4) is a receptor from the CD28 family present on activated T-cells. It inhibits T-cell maturation and differentiation by competing with the T-cell co-stimulatory receptor CD28 in binding to CD80 and CD86 ligands. Its high expression has been demonstrated on CD4⁺ cells, CD8⁺ cells, and Treg cells. High expression of CTLA-4 on Treg cells might play a key role in the induction of immunological self-tolerance [23]. Around 80% of AML samples express CTLA-4 [24]. Preclinical studies have demonstrated that CTLA-4 blockade enhances cytotoxic T-cells function and increases the proportion of T-cells producing IFN-γ [25]. In murine models, CTLA-4 blockade has triggered a graft-versus-leukemia (GVL) effect after allo-HSCT but without leading to graft-versus-host disease (GVHD) [26].

Ipilimumab

Ipilimumab is the first FDA-approved CTLA-4 inhibitor. It is indicated as monotherapy or in combination with nivolumab for melanoma. Additionally, when combined with nivolumab, it is approved for the treatment of several other malignancies including renal cell carcinoma, colorectal cancer,

non-small cell lung cancer, hepatocellular carcinoma and head and neck squamous cell carcinoma [27]. Data on its *in vivo* activity in AML is still very limited. Davids et al. [28] investigated the response to single-agent ipilimumab therapy in patients with relapse after allo-HSCT in a phase I/Ib multicenter study (median age 58 years, range 22–75). Two out of twenty-two patients had a partial response, and 5/22 had a complete response, with four of them experiencing a durable response for more than 12 months. In another clinical study, ipilimumab showed limited activity in patients after HMA failure (mean age, 67.3, standard deviation 7.9 years). One patient (3.4%) achieved a marrow complete response for three months and seven patients (24%) achieved stable disease lasting more than 46 weeks [29]. Another open-label study assessed the efficacy of ipilimumab in a group of 29 patients with relapse of solid and hematological malignancies after allo-HSCT (median age 43 years, range 21–65). No disease regression was observed in AML patients [30].

The combination therapy of ipilimumab with HMA shows more promising results. In a multicenter phase I trial using ipilimumab and decitabine for patients with relapsed and/or refractory myelodysplastic syndrome (R/R MDS) or AML before and after allo-HSCT, Garcia et al. [31] reported an OS rate of 52% in allo-HSCT-naïve patients and of 20% in patients post allo-HSCT.

PD-1/PDL-1 blockade

Programmed cell death protein 1 (PD-1) is a type I trans-membrane protein primarily expressed in activated immune cells. It binds to the programmed cell death ligand 1 (PD-L1) and PD-L2. The expression of these ligands is regulated by pro-inflammatory cytokines involving IFN-γ, IL-12 and TNF-α [32]. The pathway's role is to inhibit immune responses. Even though PD-L2 has a greater affinity to PD-1 than to PD-L1, its role in immune regulation is smaller since PD-L1 is

present in many tissue types, while PD-L2 is found mostly in hematopoietic cells [33–35]. In patients with malignancies, an overexpression of PD-L1 molecules on the cells' surface of many tumors and PD-1 receptors on T-cells infiltrating the tumor has been observed. Interaction of PD-1 with PD-L1 on tumor cells inhibits apoptosis of malignant cells and suppresses the antitumor T-cell response by inducing their exhaustion and apoptosis. Blockade of this interaction restores T-cell activity and prevents the immune escape of neoplastic cells. These discoveries have allowed for the development of effective targeted therapies for multiple malignancies [36].

In AML, overexpression of PD-1 receptors has been observed in all T-cell populations [37]. The upregulation is more frequent in patients with relapses than in newly diagnosed patients [38], and also more frequent in non-respondents than in patients with complete remission [21]. Preclinical studies have demonstrated that disease progression leads to a higher proportion of Treg cells and increased PD-1 expression in CD8+ T-cells in murine AML models. PD-1 blockade, coupled with Treg lymphocyte depletion, prolonged the survival of the mice [39, 40]. In newly diagnosed AML patients, the modulation of PD-1/PD-L1 axis by combining anti-PD-1 and anti-PD-L1 antibodies with cytarabine was shown to enhance activation of Th and Tc cells, and thus positively affect the formation of an anti-cancer immune microenvironment, without influencing the blast cells [41].

Several anti-PD-1 antibodies (nivolumab, pembrolizumab, cemiplimab) and PD-L1 inhibitors (atezolizumab, avelumab and durvalumab) have been approved for various solid tumors, but their therapeutic use in hematological malignancies, including AML, is still under examination [42].

PD-1 inhibitors

Nivolumab

In 2016, Dayer et al. [43] conducted a phase Ib/II study of the combination of nivolumab and azacitidine (AZA) with 51 enrolled patients who had failed prior therapy (median age 69 years, range 45–90). The median OS of the patients was 9.3 months, which compared favorably to the survival of similar patients treated only with AZA as salvage therapy [43].

In 2019, the same authors reported a phase II study in which 70 patients with R/R AML were treated with AZA and nivolumab (median age 70 years, range 22–90). In this trial, 65% of the patients had received prior HMA therapy. An overall response rate (ORR) of 33% was reported, involving 22% of CR or complete remission with incomplete count recovery (CRi) (four CR and 11 CRi). However, 58% of patients did not respond to the therapy. Prior HMA did not improve the outcome. The achieved ORR, CR/CRi and OS were significantly higher than in a historical cohort treated

with HMA salvage therapy. In non-responders, CTLA-4 up-regulation in CD4+ and CD8+ T-cell populations occurred, suggesting a role played by this mechanism in resistance to PD-1 blockade [44].

To further activate T-cell response, a second cohort of 31 patients was treated by the same authors with AZA, nivolumab, and ipilimumab (median age 71 years, range 26–86). The OS of 10.5 months was favorable compared to the first cohort and historical HMA-based clinical trial controls [45]. Due to the encouraging results, the authors are currently conducting phase Ib trials to study the efficacy of nivolumab and ipilimumab in patients with high-risk R/R AML or MDS after allo-HSCT (NCT03600155).

Another phase II study assessed the combination of nivolumab and induction chemotherapy with idarubicin and cytarabine in patients with newly diagnosed AML ($n = 42$) or high-risk MDS (HR-MDS) ($n = 2$) (median age 54 years, range 26–66). The median OS of all patients and of those who underwent allo-HSCT was 18.5 months and 24 months, respectively. However, there was no difference between patients who continued the therapy after remission and those who bridged to allo-HSCT, suggesting nivolumab's ability to restore antitumor immune activity. The ORR was 78% including 64% of CR and 14% of CRi. The therapy was well tolerated, and no excess immune-related adverse events (irAEs) and mortality were reported [46].

Liu et al. [47] studied the potential of nivolumab alone to eliminate MRD and as maintenance therapy in patients with AML after chemotherapy in a multi-center phase II study. Eighty patients were randomized to a treatment or a placebo arm (mean age 64.4 years, range 29–80). The 2-year PFS was identical (30%) in both arms. Nivolumab also failed to improve the 2-year OS, which was 60% in the treatment arm and 54% in the observation arm ($p = 0.23$). AEs were more common in the nivolumab arm, although they were expected and manageable. The authors concluded that patients with AML after chemotherapy did not benefit from nivolumab on its own [47].

Pembrolizumab

The therapeutic use of pembrolizumab combined with AZA was investigated in a phase II study in patients with R/R and newly diagnosed AML. In the first cohort of 37 R/R patients (median age 65 years, range 19–83), 29 (78%) were eligible for response evaluation. Among them, 14% achieved CR/CRi and 4% achieved PR. The median OS was 10.8 months. In the second newly diagnosed cohort, 8/17 evaluable patients (median age 75 years, range 67–83) showed CR/CRi (47%), 2/17 showed PR (12%), and the median OS was 13.1 months. In the first cohort, 24% of patients showed grade 3/4 irAEs, while in the second cohort, they were observed in 11% of patients [48].

Goswami et al. [49] tested the potential synergy of pembrolizumab and decitabine combination therapy in R/R

AML patients. The cohort included 10 previously treated patients (median age 62 years, range 30–81). One patient achieved a morphological leukemia-free state. Three had stable disease, and two were in CR. The median OS was 10 months. The AEs were consistent with a single-agent decitabine therapy, except for two patients who experienced irAEs [49].

Pembrolizumab was also tested in combination with HiDAC to examine whether PD-1 inhibition improves the outcomes of intensive chemotherapy in R/R AML. The ORR, CR rate, and median OS of the 37 enrolled patients were 46%, 38%, and 11.1 months, respectively (median age 54 years, range 24–70). Both patients with R/R AML and those receiving the treatments for the first time had good outcomes (13.2 and 11.3 months of median OS, respectively). The irAEs were, in general, self-limiting and easily resolved [50]. Nine of the examined patients were bridged to allo-HSCT. In a retrospective analysis, they were compared to a historical group of 18 AML patients after allo-HSCT without prior ICI therapy. OS was comparable between the two groups (67% vs. 78%). However, in contrast to the control cohort, the ICI group showed no 100-day mortality (0% vs. 17%) and no chronic GVHD [51].

No publication on cemiplimab in AML has yet been released. One study is currently recruiting (NCT03017820).

PD-L1 inhibitors

Durvalumab

A randomized, open-label study investigated the combination of AZA and durvalumab in HR-MDS and AML patients (median age 76 years, range 65–89). Both AML and HR-MDS patients were randomized into two arms to receive either AZA and durvalumab, or AZA alone. The difference in ORR between the arms in either cohort was insignificant. The authors concluded that the combination of drugs does not bring significant clinical benefits compared to AZA alone [52].

Another study, FUSION-AML-001, compared AZA with or without durvalumab as a first-line treatment for patients with AML or HR-MDS. Patients with AML were enrolled in two arms of 64 and 65 patients, respectively. In both arms, ORR, OS, and duration of response were similar [53]. The HR-MDS cohort was divided into two arms, each of 42 patients, with no significant differences in ORR and median OS between them [54].

A study assessing the efficacy and safety of the combination of AZA and durvalumab in previously untreated HR-MDS or AML elderly patients has been completed (NCT02775903). No studies are currently recruiting.

Avelumab

Zheng et al. [55] conducted a phase I study to assess the safety and tolerability of the combination of avelumab and decitabine in patients with untreated AML unfit for HiDAC.

Seven patients were enrolled (median age 72 years, range 62–78). Two patients died of sepsis before the end of therapy, meaning that five patients were evaluated for response. One achieved CR, one showed disease progression, and the remaining three had stable disease. The median OS was 3.2 months. Regarding AEs, two patients developed grade three pneumonitis and five patients died of septic shock. The authors concluded the therapy did not bring about a clinical benefit and contributed to a significant increase in sepsis-related deaths [55].

The efficacy of avelumab with AZA in patients with R/R AML was assessed in a phase Ib/II clinical trial. Nineteen patients were treated (median age 66 years, range 22–83), 66% of whom had prior exposure to HMA. Two patients achieved CRi. The median OS was 4.8 months. The efficacy was comparable to the historical CR/CRi rate of 16% and the median OS of 6.7 months in a large cohort of R/R AML patients treated with AZA only. Mass cytometry performed in the same study revealed a higher expression of PD-L2 compared to PD-L1 on AML blasts with increasing PD-L2 expression during therapy, suggesting its role in AML immune escape [56]. No new studies are recruiting at the moment.

Atezolizumab

The safety of a combination of atezolizumab and gilteritinib (an FMS-like tyrosine kinase 3 inhibitor) was assessed in a phase I dose-escalation study by Altman et al. [57]. Eleven adults with relapsed or refractory FLT3-mutated AML were recruited (median age 82 years, range 68–84). Serious treatment-related AEs were reported in 10 patients (90.9%) and led to the withdrawal of study treatment in eight. In three patients, they led to death. The authors concluded that the combination had an acceptable safety profile with no new safety signals from either of the agents [57].

Another phase Ib study by Prebet et al. [58] evaluated the safety and efficacy of the combination of atezolizumab with guadecitabine in patients with R/R AML or newly diagnosed AML. All sixteen enrolled patients (median age 73 years, range 43–82) reported at least one AE and 15/16 reported an AE of grade 3 or higher. Eight patients died due to disease progression and six due to AEs. One patient achieved CRi. The overall benefit-risk profile of the drug combination was unfavorable [58].

Several clinical trials investigating the safety of drug combinations involving atezolizumab have been completed lately (NCT03390296, NCT03730012, NCT02892318).

TIM-3/Galectin-9 axis

T-cell immunoglobulin and mucin domain-containing protein 3 (TIM-3) is a type I transmembrane protein found in various immune cells. It plays a crucial role in inhibiting Th1 responses and expressing inflammatory cytokines

such as TNF- α and IFN- γ . The ligand with the highest affinity to the TIM-3 domain is a soluble Galectin-9 (Gal-9). Their interaction triggers cell death in effector Th1 cells, reducing inflammation and promoting tumor cell evasion in malignant diseases [59]. In AML patients, TIM-3 expression on peripheral T cells significantly increases and positively correlates with a worse prognosis [60, 61]. In murine models after xenotransplantation, anti-TIM3 monoclonal antibodies eliminated LSCs able to reconstitute human AML [62]. Several anti-TIM3 monoclonal antibodies have been synthesized, but only MGB453 (sabatolimab) has demonstrated preliminary safety and efficacy in the context of AML [63]. Sabatolimab is a high-affinity, humanized IgG4 monoclonal antibody targeting TIM-3 on immune and leukemic cells [64].

The safety and response rate of the combination of sabatolimab and HMA in AML and HR-MDS were evaluated in a phase I study by Brunner et al. [66]. Fifty AML patients received sabatolimab + decitabine. Sixteen AML patients received sabatolimab + AZA. In the sabatolimab + decitabine cohort, three patients experienced irAEs of grade 3 or higher. In the sabatolimab + AZA cohort, no irAEs of grade 3 or higher were reported. For sabatolimab + decitabine, ORR was 41% and 24% in ND-AML and R/R-AML respectively. For MGB453 + AZA, ORR was 27% for ND-AML. Results for both combinations were encouraging [65]. A subsequent study discovered that R/R AML and HR MDS patients treated with the combinations of sabatolimab and HMA have favorable outcomes after HCT [66].

Based on these promising results, a STIMULUS clinical trial program was initiated to evaluate multiple combination therapies with sabatolimab in AML, HR-MDS and CMML during phase II and III trials. STIMULUS-AML1 is a phase II, single-arm study of sabatolimab, venetoclax and AZA in newly diagnosed AML patients with a median age of 77 years. The interim results showed the safety profile was comparable to that of venetoclax + AZA therapy [67]. The STIMULUS-AML2 study tested sabatolimab (400 mg or 800 mg IV) alone or in combination with AZA for AML patients with MRD detected after allo-HSCT. Twenty-one patients with a median age of 59 years were enrolled, 10 at 400 mg and 11 at 800 mg. At data cut-off, in the 400 mg cohort, three patients were still ongoing, and seven had to discontinue due to relapse. In the 800 mg cohort, seven patients had to discontinue: due to relapse (five), AE (one), or new therapy (one). No cases of GvHD or irAEs were reported in either cohort [68].

LAG-3/MHC signaling

LAG-3 is a type I transmembrane glycoprotein with a similar domain structure to CD4 [69]. It binds to MHC class II with a higher affinity than CD4, downregulating T-cell activation, proliferation, and cytokine production [70]. LAG-3 also

binds to galectin-3, a lectin-modulating T-cell activity that is expressed in many solid tumors. The interaction between LAG-3 and galectin-3 inhibits CD8 (+) effector T cell function by lowering levels of plasmacytoid dendritic cells [71]. Another MHC-II independent, functional ligand for LAG-3 is liver-secreted fibrinogen-like protein 1 (FGL1). Blockade of their interaction by monoclonal antibodies stimulates immune reaction to tumor cells [72]. High expression of LAG-3+ T cells has been found in patients with newly diagnosed AML [73]. LAG-3/PD-1 co-expression in AML correlates with poor OS and might be used as a potential target for novel therapies [74]. In an *in vitro* model, LAG-3 blocking antibodies diminished immune evasion of AML cells by increasing T cell activation, lowering the number of Tregs, and improving MHC-I mediated toxicity against tumor cells [75]. Currently, one clinical trial is investigating the use of the combination of 5-AZA, nivolumab and an anti-LAG-3 antibody *i.e.* relatlimab for the treatment of patients with R/R AML and older patients with newly diagnosed AML (NCT04913922).

CD27/70 interactions

CD27 and CD70 are members of the tumor necrosis factor superfamily. CD27 is generally found in naïve T, memory T cells, B cells, and NK cells. CD70, on the other hand, is only transiently expressed on activated cells. Upon binding to CD70, CD27 interacts with TNF receptor-associated factors (TRAFs), leading to activation of T, B, and NK cells through NF- κ B. However, chronic triggering of CD27 leads to T-cell exhaustion [76]. In hematological malignancies, CD70 may be aberrantly expressed in tumor cells with co-expression of CD27. Riether et al. [77] investigated CD70/CD27 signaling in AML as a potential therapeutic target. They discovered that CD27 and CD70 were constitutively co-expressed on LSCs in contrast to healthy HSCs. Soluble CD27 was also significantly elevated in the serum of AML patients and proved to be an independent negative prognostic factor. It was also discovered that CD70/CD27 signaling triggers the Wnt pathway in AML, promoting tumor progression. Attempts to block the CD70/27 interaction in AML stem cells resulted in cell growth inhibition and differentiation induction *in vitro*. In AML xenotransplanted mice, blocking the CD70/CD27 interaction prolonged survival [77]. Preclinical trials showed that leukemia stem cells in AML upregulate CD70 in response to HMA treatment, leading to increased CD70/CD27 signaling. Targeting CD70-expressing LSCs with a human α CD70 monoclonal antibody with enhanced antibody-dependent cellular cytotoxicity activity (cusatuzumab) eliminated LSCs *in vitro* and in xenotransplantation experiments.

Based on these findings, Pabst et al. [78] performed a phase I/II trial in previously untreated older patients with AML with a single dose of cusatuzumab monotherapy

followed by combination therapy with AZA. Initial results show hematological response in all 12 patients, with a CR in eight of them and complete remission with Cri in two (median age 75 years, range 64–84). No dose-limiting toxicities were reported [78]. Due to the promising results, a phase II study of cusatuzumab + AZA was started in patients with newly diagnosed AML ineligible for intensive chemotherapy (NCT04023526). Another phase Ib study evaluating the combination of cusatuzumab with venetoclax and AZA in patients with AML not eligible for intensive chemotherapy is active but not recruiting (NCT04150887).

SIRPα/CD47 pathway

CD47 is a transmembrane protein expressed on the surface of every cell type, and its primary function is to inhibit phagocytosis. When CD47 binds to signal regulatory protein alpha (SIRPα) present on macrophages, it sends a “don’t eat me” signal [79]. The level of CD47 expression is higher in LSC in AML than in normal HSCs, and has been proven to be a negative prognostic factor [80, 81]. Chao et al. [82] identified calreticulin as a highly pro-phagocytosing signal expressed on LSCs but minimally on normal cells. Calreticulin expression in cancer cells correlates strongly with CD47 expression, suggesting the protective role of increased CD47 from calreticulin-mediated phagocytosis of cancer cells. This leads to high susceptibility of LSCs to anti-CD47 antibodies [82]. It has been shown that anti-CD47 antibodies enable phagocytosis of AML cells *in vitro* and eradicate human AML in xenografted mice, indicating the high potential of this new therapeutic agent [83].

Magrolimab

Magrolimab, previously known as 5F9, is a first-in-class humanized anti-CD47 antibody that enhances tumor cell phagocytosis by blocking CD47/SIRPα interaction. Despite promising results of many studies investigating its therapeutic use in AML, magrolimab has demonstrated both futility and an increased risk of death in a population of patients with hematological malignancies. Therefore, the US Food and Drug Administration (FDA) has halted all clinical studies of magrolimab in AML and MDS [84].

Evorpaccept

Evorpaccept (ALX148) is a high-affinity CD47-blocking protein with an inactive modified Fc domain to prevent phagocytosis of red blood cells and thus minimize toxicity. There are currently two studies investigating evorpaccept in AML. The ASPEN-05 phase I/II open-label multicenter study is evaluating the safety and tolerability of evorpaccept in combination with standard venetoclax and AZA in AML. The results from phase Ia show that the combination is well-tolerated without dose-limiting toxicities. In all 12 assessed patients (median age 74 years, range 56–82), a reduction

in bone marrow blasts has been observed [85]. The results of the second ongoing trial evaluating evorpaccept + AZA for HR-MDS (NCT04417517) are yet to be published.

Lemzoparlimab

Lemzoparlimab is a second-generation anti-CD47 IgG4 antibody with a distinctive binding epitope that reduces the risk of anemia. A phase I/II study of lemzoparlimab as monotherapy in patients with AML or MDS has been completed. Four out of five patients developed treatment-emergent AEs, but only one of them developed a grade 3 AE [86]. A phase III trial of lemzoparlimab with AZA for previously untreated HR MDS patients is currently recruiting (NCT05709093).

Conclusions

Despite significant advances in the treatment of AML, it continues to be characterized by low rates of survival and high rates of relapse. Even though ICIs have brought long-lasting responses in many malignant diseases, none have yet been approved for AML treatment. Classical CTLA-4 and PD-1 inhibitors represent a promising frontier in AML treatment, but their clinical application is still limited by the mixed results obtained in clinical trials, with significant AEs in some cases.

However, the optimistic outcomes of combination therapies of ICIs and HMA encourage further research in this area. Furthermore, numerous ongoing clinical trials are evaluating the therapeutic potential of novel targets such as TIM-3 or LAG-3, and the synergistic effect of drug combinations, bringing optimism to the future perspectives of AML therapy.

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Treatment options for anaemia in myelofibrosis

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Introduction

Primary myelofibrosis (PMF) is one of the myeloproliferative neoplasms (MPN) without the BCR-ABL fusion gene [1]. This group also includes polycythemia vera (PV) and essential thrombocythemia (ET) [2]. Myelofibrosis can also result from myelofibrotic transformation of both PV and ET (when it is known as post-PV-MF, post-ET-MF) [2]. MF carries the worst prognosis among the Ph-negative MPNs, primarily due to the increased risk of transformation to acute leukaemia [1, 3]. The cause of MF remains largely unknown. The leading mutations found in most patients are mutations in the *JAK2*, *CALR* and *MPL* genes. All of these cause constant activation of the JAK-STAT signalling pathway, resulting in excessive proliferation of megakaryocytes, which secrete growth factors that stimulate the activity of fibroblasts in the bone marrow (BM) and numerous proinflammatory cytokines [4]. Myelofibrosis is usually accompanied by constitutional symptoms (including fatigue, weight loss, fever, bone pain), splenomegaly and anaemia [1]. Anaemia may result from the underlying disease (MF-related anaemia) or can be a side effect of therapy (treatment-related anaemia). Regardless of the cause, anaemia significantly worsens patients' quality of life, and in addition, MF-related anaemia is a poor prognostic factor [5–7].

Current treatment options for MF-related anaemia include erythropoiesis-stimulating agent (ESA), androgens (danazol), glucocorticoids, and immunomodulatory drugs (IMiDs). All of them are characterised by limited efficacy and durability of response, and in many cases packed red blood cells (RBCs) transfusions remain the only effective therapy [8]. In recent years, thanks to progress in the understanding of the pathogenesis of MF, many new

therapies have been developed aimed not only at reducing the spleen volume and general symptoms, but also at stimulating erythropoiesis. These include new JAK inhibitors, e.g. pacritinib and momelotinib, as well as drugs with other targets such as luspatercept, imetelstat, and pelabresib. This review article discusses the pathogenesis and clinical significance of anaemia in MF and presents new treatment options.

Epidemiology of MF-related anaemia

Moderate anaemia, defined as when the haemoglobin (Hb) level is less than 10 g/dL, occurs in c.35% of MF patients, and nearly one quarter of patients require RBC transfusions [6, 9]. In Barraco et al.'s [10] study including 722 patients with PMF, as many as 87% had anaemia at diagnosis: 37% severe (Hb <8 g/dL), 16% moderate (Hb 8–10 g/dL), and almost half mild anaemia (below the gender reference range, but above 10 g/dL). In another retrospective analysis [11] including 1,109 patients, a similar rate of patients with anaemia was found, with a median Hb level at diagnosis of 10.1 g/dL (range: 5–16.7). Regardless of the severity, anaemia was significantly more common in women than in men. Moderate or severe anaemia was found more frequently in patients >65 years (56% and 65%, respectively) and with more advanced disease [11].

In patients with secondary myelofibrosis, the median Hb level at diagnosis is slightly higher (c.11 g/dL), albeit with increasingly severe anaemia over time [12]. Hb levels are lower in patients with post-ET MF than in patients with post-PV MF, and they correlate with the grade of bone marrow fibrosis [13, 14].

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Prognostic significance of anaemia

Anaemia is the main symptom of MF in addition to constitutional symptoms and splenomegaly. Its presence significantly worsens patients' quality of life, not only due to the burden of symptoms, but also because of the requirement for periodic hospitalisation to allow the RBC transfusion [15]. In today's era of JAK2 inhibitors, which significantly reduce splenomegaly-related and general symptoms, anaemia is the most important factor worsening quality of life. Additionally, anaemia affects the prognosis of patients with MF. The significance of anaemia as a prognostic factor is underlined by the fact that it is included in all prognostic scoring systems used in MF patients, both the first ones, based only on clinical data such as the International Prognostic Scoring System (IPSS) and Dynamic IPSS (DIPSS), as well as more modern ones which also take into account molecular and cytogenetic aberrations, e.g. MIPSS70 (Mutation-enhanced IPSS) and DIPSS plus [1, 6, 7, 16, 17]. In the DIPSS index, which assesses prognosis already during the course of the disease, anaemia <10 g/dL is the only factor assigned 2 points, and in the DIPSS plus index, RBC transfusions-dependence is additionally specified [6, 7].

Pathogenesis of MF-related anaemia

The pathogenesis of MF-related anaemia is very complex, with different dominant processes at different stages of the disease, and it is still not fully understood. The primary process underlying the development of anaemia is the gradual displacement of erythropoiesis from the bone marrow resulting from fibrosis [18]. This in turn leads to the activation of extramedullary haematopoiesis, among others locations in the spleen. It is currently believed that extramedullary haematopoiesis in the spleen is associated with abnormal transport of BM-derived clonal progenitor cells and stem cells as a result of dysregulation of the cytokine balance in the bone marrow [19]. Haematopoietic cells proliferate and alter splenic microarchitecture, leading to splenomegaly [20]. Haematopoiesis in the spleen is unable to provide an adequate number of erythrocytes, which results in anaemia. The enlarged spleen excessively sequesters and destroys circulating erythrocytes, contributing to increased anaemia severity [21]. Additionally, processes similar to those in anaemia in the course of chronic diseases associated with excessive expression of hepcidin, the main protein regulating iron absorption, play an important role in the development of MF-related anaemia. In MF, an increased hepcidin level results from increased secretion of inflammatory cytokines, especially interleukin 6 (IL-6) and excessive activation of the BMP6/ACVR1/SMAD (bone morphogenetic protein/activin receptor/Smad protein) pathway. Bone morphogenetic proteins are pleiotropic cytokines belonging to the transforming growth factor

beta (TGF β) superfamily. They are bound by two different types of receptors with serine-threonine kinase activity, and the second messengers of the intracellular signal are Smad proteins [22]. Pathogenic mechanisms of MF-related anaemia are presented schematically in Figure 1.

Anaemia in MF patients may also be iatrogenic and occur as a result of treatment, including JAK inhibitors or hydroxyurea (HU). In addition to the above-mentioned causes, there are also other possible factors leading to the development of anaemia in MF patients, e.g. concomitant chronic diseases, chronic blood loss (gastric/duodenal ulcer, oesophageal varices, haemolysis), and iron or vitamin deficiency. In the differential diagnosis of new-onset or worsening anaemia, causes not directly related to MF should always be considered.

Treatment of MF-related anaemia

MF-related anaemia is a therapeutic challenge. Currently, the options used in clinical practice include RBC transfusions, ESAs, androgens (danazol), glucocorticoids, and IMiDs. Their efficacy and influencing factors are set out in Table I. Due to differing patient populations and differing definitions of treatment response, direct comparisons between the clinical trials listed in Table I and assessments of the efficacy of different treatment options are not possible. For clinical trial purposes, the International Working Group for Research and Treatment of Myeloproliferative Neoplasms (IWG-MRT) and the European LeukaemiaNet have standardised the definition of transfusion dependency (TD) in MF patients: it consists of a requirement for transfusion of ≥ 6 RBC units within 12 weeks prior to study entry, with Hb level <8.5 g/dL, in the absence of bleeding or treatment-related anaemia, and the last transfusion coming within 28 days of study entry. Transfusion independence (TI) is classified as the absence of RBC transfusions during any 12-week treatment period (in the study) while maintaining Hb level >8.5 g/dL [23].

Erythropoiesis-stimulating agent (ESAs)

Erythropoiesis-stimulating agents activate proerythroid signalling, resulting in increased erythrocyte production. Recombinant human erythropoietin (rhEPO) and darbepoetin α are used in the treatment of MF-related anaemia. Prospective clinical trials with ESAs involved a small number of patients, and the response to treatment ranged between 20% and 45% [24, 25]. In retrospective studies, the differences in the efficacy of ESAs were even greater, and some did not prove their efficacy in any patient [26]. Predictive factors of response to ESA therapy include low baseline endogenous erythropoietin (EPO) serum activity and low transfusion requirements. However, even in patients eligible for treatment, the response rate is not predictable and patients will eventually become refractory

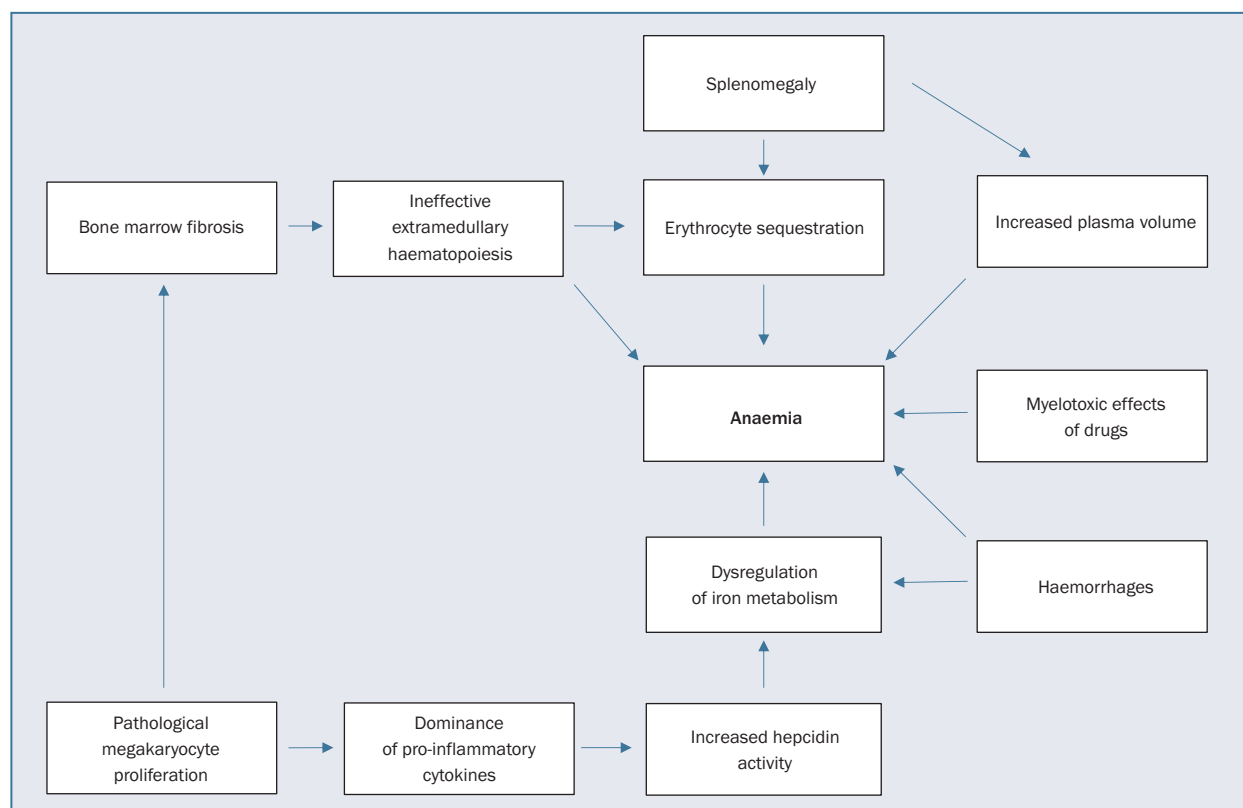


Figure 1. Causes of anaemia in myelofibrosis. Developed based on [21]

to ESA therapy [27]. The main limitations of ESA use in MF patients are thus their limited efficacy in transfusion-dependent patients, the risk of progression of splenomegaly, and thrombotic complications [1]. In Poland, EPO is not reimbursed in patients with MF.

Androgens

Androgens have erythropoiesis-stimulating properties that are used in the treatment of MF-related anaemia, although their exact mechanism of action is not fully understood [28]. Initially, testosterone enanthate and oral fluoxymesterone were used, but due to a better safety profile and tolerability, only danazol, a synthetic, attenuated androgen, is now used [29]. The recommended dose is 600 mg/day. The response rate is c.30%, including 44% in patients who are transfusions independent and 19% in transfusions-dependent patients [29]. The most common adverse effect of danazol is increased liver enzyme activity, which usually resolves after dose reduction. Danazol cannot be used in patients with androgen-dependent tumours such as prostate cancer, previous or active thrombosis, or significantly impaired liver or kidney function.

Immunomodulatory drugs (IMiDs)

The first report of the efficacy of this group of drugs concerned thalidomide, which was used in a patient with

very advanced MF. The result was a significant reduction in spleen size and bone marrow fibrosis after 30 months of therapy [30]. The positive effect of IMiDs in reducing anaemia, thrombocytopenia and, to a lesser extent, splenomegaly in patients with MF results from the broad anti-inflammatory, antiangiogenic, antiproliferative and immunomodulatory effects of this group of drugs [31]. In clinical studies, IMiDs have been used either as monotherapy or in combination with glucocorticoids (see Tab. I). Small sample sizes, the addition of other drugs, as well as different inclusion criteria and ways of assessing responses make it difficult to assess their true effectiveness. In the initial studies, thalidomide was used in high doses (400–500 mg/d), which showed efficacy in reducing anaemia, but the treatment was swiftly discontinued due to high toxicity, mainly polyneuropathy. Subsequent studies have shown that doses of 50–100 mg/d allow for the maintenance of treatment response with significantly lower toxicity [32]. Studies have shown the efficacy of low-dose thalidomide in combination with prednisone in c.40% of cases, with a median duration of response (DoR) of 16 weeks [27]. In some patients, even such low doses of thalidomide (50–100 mg/d) can cause fatigue, constipation, and neuropathy.

In Mesa et al.'s [33] phase II study with lenalidomide, a second-generation IMiD, the response rate was c.20% when the drug was used as monotherapy and 30% when

Table I. Options for treating anaemia in patients with myelofibrosis

Drug/method	Study	Study population	Indications	Results	Factors influencing treatment response
ESAs	Cervantes [24]	n = 20 Median Hb 8.9 g/dL	Hb <10 g/dL or transfusion dependence	Response rate: 45%: 20% TI 25% reduction in transfusion requirements	Transfusion independence Epo activity <125 U/L
	Hernández-Boluda [25]	n = 163 Median Hb 9.3 g/dL	Hb <10 g/dL Epo <125 U/L	Response rate: 53%: 29% in TD patients, 53% in TI patients	Female gender Leukocytes >10 G/L Ferritin <200 ng/mL
Androgens (danazol)	Cervantes [29]	n = 50 Median Hb 8.5 g/dL	Hb <10 g/dL	Response rate: 30%: 19% in TD patients 44% in TI patients	
IMiDs					
Thalidomide vs. placebo	Abgrall [72]	n = 52 Median Hb 8.8 g/dL	Hb <9 g/dL or transfusion dependence	Response rate: 17% (thalidomide) vs. 16% (placebo) Reduction in transfusion requirements 16% vs. 26%	Not found Only 10 patients completed six months of treatment High treatment toxicity
Lenalidomide + prednisone	Mesa [33]	n = 48 Median Hb 9.1 g/dL	Hb <10 g/dL or transfusion dependence	Response rate: 19%	Not found. 35% of patients did not complete treatment. High haematological toxicity.
Pomalidomide vs. placeboRE-SUME study	Tefferi [36]	n = 229 Median Hb 8.7 g/dL	Hb <9 g/dL or transfusion dependence	Response rate: 16% (pomalidomide) vs. 16% (placebo)	Transfusions <4 RBC units/month Age <65 Primary myelofibrosis
Splenectomy	Tefferi [39]	n = 223 Median Hb 10.4g/dL	Splenectomy for anaemia or symptomatic splenomegaly	37% in TD patients	PLT >50 G/L High cellularity of bone marrow
RBC transfusions	Elena [41]	n = 220 Median Hb 11 g/dL 14% TD (Median Hb 7.1 g/dL)	Comparison of prognosis of TD to TI patients	NA	Transfusion dependency shortens overall survival

Epo – erythropoietin; ESA – erythropoiesis stimulating factors; Hb – haemoglobin; IMiDs – immunomodulatory drugs; NA – not applicable; PLT – platelet count; RBC – red blood cells; TD – transfusion-dependent; TI – transfusion-independent

in combination with prednisone. Haematological toxicity was the main problem: the authors found the frequency of haematological toxicities to be as high as 88% despite the addition of prednisone [33]. The only comparison of thalidomide with lenalidomide to date was conducted by Jabbour et al. [34], who evaluated 125 patients treated in three consecutive phase II studies. Thalidomide was used in doses increasing up to 800 mg/d (n = 44), lenalidomide in a dose of 10 mg/d (n = 41), and lenalidomide in combination with prednisone in doses of 10 mg and 30 mg (n = 40). Lenalidomide-based therapy was more

effective (34–38%) than thalidomide (16%; $p = 0.06$). The combination of lenalidomide and prednisone resulted in a significantly longer duration of response (median 34 months) compared to either lenalidomide or thalidomide monotherapy (median 7 and 13 months, respectively; $p = 0.042$) [34].

The newest drug in this group is pomalidomide, which shows the most favourable safety profile, but data on its efficacy also differs significantly. In a phase II study with 96 patients with MF-related anaemia, in which pomalidomide was used as monotherapy, 39% of patients achieved

a response to treatment, and the median duration of response was 13 months [35]. But in a phase III study with 229 patients, the response rate in the pomalidomide arm was similar to that of a placebo [36].

To conclude, IMiDs show moderate efficacy in patients with MF-related anaemia, but the main limitation of their use is toxicity. The most common adverse events are haematological toxicity, polyneuropathy, fatigue, and constipation. Currently, studies are underway to combine IMiDs with JAK2 inhibitors. Preliminary results indicate clinical benefits and moderate toxicity with sequential use of the drugs [37, 38].

Splenectomy

Treatment-refractory symptomatic splenomegaly in MF patients may be an indication for splenectomy [1]. Splenectomy leads to the resolution of symptoms related to spleen enlargement, and reduces anaemia, thrombocytopenia, and portal hypertension, but is also associated with a significant risk of complications. In a retrospective analysis from the Mayo Clinic covering 23 patients with MF and anaemia who underwent splenectomy, 37.6% of patients became RBC transfusions independent or maintained an Hb level >10 g/dL [39]. Perioperative mortality was 9%, and complications occurred in 30% of patients, most often increased platelet (PLT) count and thromboembolic or haemorrhagic episodes. The results of splenectomy were also summarised in a more recent analysis from the same centre, including 120 consecutive patients with MF who underwent splenectomy, with median age 66 and a transfusion-dependent rate of 60% [40]. During a median post-splenectomy follow-up of 1.3 years, 95 (79%) deaths and 30 (25%) leukaemic transformations were reported. Median overall survival (OS) after surgery was 1.5 years. Factors adversely affecting survival included age >65, RBC transfusion dependence, leukocyte count >25 G/L, and peripheral blasts (PB) ≥5% [40]. Splenectomy in MF patients is mainly performed as palliative therapy, and the complete blood count (CBC) improvement is only seen in the short term. The procedure is associated with a high risk of perioperative complications, including fatal ones.

Packed red blood cells (PRBCs) transfusion

Red blood cells transfusion dependence is very common in patients with advanced MF, and its occurrence is associated with a significant reduction in overall survival [9, 41]. Complications of RBC transfusion include alloimmunisation and iron overload. Due to the expected short-term survival associated with transfusion dependence, iron chelation is usually not used unless the patient is considered a candidate for allogeneic haematopoietic stem cell transplantation (ASCT) [1]. With this in mind, one goal of MF therapy should be to maximise the time window during which the patient does not require RBC transfusions.

JAK2 inhibitor therapy and anaemia

JAK inhibitors have significantly changed the therapy landscape for patients with MF. Their use allows for reductions of general symptoms and spleen size, as well as OS prolongation [1]. Unfortunately, due to JAK-STAT pathway inhibition, which is their primary mechanism of action, they also inhibit normal haematopoiesis, which may result in new-onset or worsening of anaemia or thrombocytopenia [42]. Such results are exerted primarily by the first two registered JAK inhibitors: ruxolitinib (RUX) and fedratinib (FED). The next two, pacritinib and especially momelotinib, inhibit erythropoiesis to a much lesser extent, and improve red blood cell parameters in a significant percentage of patients due to their additional mechanisms of action. A summary of the results of pivotal studies of JAK inhibitors, including the response in terms of anaemia, is set out in Table II.

Ruxolitinib

Ruxolitinib was the first JAK inhibitor approved for the treatment of MF patients [43]. The approval was based on the results of the COMFORT I and COMFORT II studies [44, 45]. COMFORT I compared RUX to a placebo in the treatment of 309 patients with IPSS intermediate-2 or high-risk MF. The dose of RUX ranged from 15 to 20 mg depending on the baseline PLT count. The primary endpoint of this study was spleen volume reduction of at least 35% (SVR35) after 24 weeks of treatment. In the RUX arm, the endpoint was met by 41.9% of patients, compared to just 0.7% in the placebo arm ($p < 0.0001$). Additionally, 45.9% of patients had at least a 50% reduction in the total syndrome score (TSS50) [44]. The COMFORT-II study involved 219 patients in intermediate-2 and high-risk groups and compared RUX to best available therapy (BAT) [45]. The primary endpoint was the same as in COMFORT-I. After 24 weeks of treatment, 32% of patients treated with RUX achieved SVR35, compared to zero patients in the BAT arm. After 48 weeks of treatment, patients receiving RUX had significant reductions in MF-related symptoms, including loss of appetite, dyspnoea, fatigue, insomnia, and pain, whereas these symptoms worsened in patients receiving best available therapy. Assessment of constitutional symptoms and quality of life measures in this study were based on the EORTC QLQ-C30 (European Organisation for Research and Treatment of Cancer Quality of Life questionnaire core model) and FACT-Lym (Functional Assessment of Cancer Therapy–Lymphoma) scales [45].

In a 3-year pooled analysis of data from both studies, patients treated with RUX had a longer OS [46]. This was also true for patients who transferred to the RUX arm during the course of the study. In both studies, anaemia and thrombocytopenia were the most common adverse events, albeit

Table II. JAK inhibitors approved for treatment of MF

Drug	Target	Study	Population	Treatment	SVR35 at week 24	TSS50	Response in terms of anaemia	Toxicity
Ruxolitinib (RUX)	JAK1 JAK2	COMFORT-1 phase III [44]	309 MF patients Int-2 or high risk	15–20 mg BID (depending on PLT count) vs. placebo	41.9% vs. 0.7%	45.9% vs. 5.3%	TI: 41% (14/34 TD)	Grade 3/4 anaemia: (COMFORT-1: 45.2%, COMFORT-2: 42%), Thrombocytopenia Headache Opportunistic infections
		COMFORT-2 phase III [45]	219 MF patients Int-2 or high risk	15–20 mg BID (depending on PLT count) vs. BAT	32% vs. 0%	NA	NA	
Fedratinib (FED)	JAK1 JAK2 JAK3 TYK3	JAKARTA phase III [51]	289 MF patients Int-2 or high risk	400 mg/d vs. 500 mg/d vs. placebo	36% vs. 40% vs. 1%	36% vs. 34% vs. 7%	TI: 88% (7/8 TD)	Grade 3/4 anaemia: FED 400: 43% FED 500: 60% Placebo: 25% JAKARTA-2: 38%. Increased transaminase, lipase and amylase activity. Wernicke's encephalopathy
		JAKARTA-2 phase II [52]	97 MF patients Int-1 with symptoms, int-2 or high risk RUX resistance/ intolerance	400 mg/d	30%	27%	NA	
		FREEDOM [53]	38 MF patients Int or high risk according to DIPSS RUX resistance/ intolerance	400 mg/d	9%	16%	NA	
		FREEDOM2 [54]	201 MF patients Int-2 or high risk RUX resistance/ intolerance	400 mg/d vs. BAT	36% vs. 6%	NA	NA	
Pacritinib (PAC)	JAK2 ACVR1 IRAK1 FLT3 CSF1R	PERSIST-1 phase III [73]	327 MF patients Int-1, int-2 or high risk JAK inhibitor naïve vs. BAT (excluding RUX)	400 mg/d	19%	19%	TI: 25% (9/36 TD)	Thrombocytopenia Anaemia Diarrhoea Fluid retention Heart failure Squamous cell carcinoma of skin
		PERSIST-2 [61]	311 MF patients Int-1, int-2 or high risk Regardless of JAK inhibitor exposure PLT count <100 G/L vs. BAT (including 45% with RUX)	400 mg/d or 200 mg BID	18%	25%	TI ≥8 weeks Hb increase ≥2 g/dL: 25% (11/44 with Hb <10 g/dL)	



Table II. (cont.) JAK inhibitors approved for treatment of MF

Drug	Target	Study	Population	Treatment	SVR35 at week 24	TSS50	Response in terms of anaemia	Toxicity
Momelotinib	JAK1 JAK2 ACVR1	SIMPLIFY-1 [57]	432 MF patients Int-1 with symptoms, int-2 or high risk PLT count ≥50 G/L JAK inhibitor naïve vs. RUX	200 mg/d	26.5%	28.4%	Ti: 66.5%	Anaemia Thrombocytopenia Neutropenia Increased transaminase, lipase and amylase activity.
		SIMPLIFY-2 phase III [58]	156 MF patients Int-1 with symptoms, int-2 or high risk RUX resistance/ /intolerance vs. BAT (including 89% with RUX)	200 mg/d	7%	26.2%	Ti: 43%	Peripheral polyneuropathy 1 st dose effect (transient hypotension, flushing, nausea, dizziness)
		MOMENTUM phase III [59]	195 MF patients Int-1, int-2 or high risk with symptoms PLT count ≥25 G/L Hb <10 g/dL Prior treatment with JAK inhibitor vs. danazol	200 mg/d	23%	25%	Ti: 31%	

ACVR1 – activin A receptor type I; BAT – best available therapy; BID – twice daily; CSF1R – colony stimulating factor 1 receptor; DIPSS – Dynamic International Prognostic Scoring System; FED – fedratinib; Hb – haemoglobin; int-1 – intermediate risk-1; int-2 – intermediate risk-2; IRAK1 – interleukin-1 receptor-associated kinase 1; JAK – Janus kinase; MF – myelofibrosis; NA – not applicable; PAC – pacritinib; PLT – platelets; RUX – ruxolitinib; SVR35 – reduction in spleen size by ≥35%; TD – transfusion dependent; Ti – transfusion independent; TSS50 – 50% reduction in severity of symptoms on TSS (total syndrome score); TYK – tyrosine kinase

with a low discontinuation rate [44, 45]. In the COMFORT I study, grade ≥3 anaemia occurred in 45.2% of patients in the RUX arm compared to 19.2% in the placebo arm [44]. The frequency of this complication in the COMFORT II study was similar in the RUX arm (42%) but higher in the BAT arm (31%) [45]. Gupta et al. [47] conducted a post-hoc analysis of both COMFORT studies to determine the long-term effects of anaemia caused by RUX treatment. This analysis showed that 61% of patients who did not have anaemia at baseline developed it during the treatment, while 69% of patients with anaemia at baseline experienced its worsening. In both cases, anaemia did not adversely affect OS [47]. The nadir of Hb level was observed after 8–12 weeks of treatment, after which Hb level returned to close to baseline values [44, 45, 47]. Nevertheless, anaemia during RUX treatment is a significant clinical challenge. In real-world studies, it has been the reason for RUX therapy discontinuation in c.10% of patients [48]. RUX-related anaemia has been successfully managed with ESA or a dose reduction below that based on PLT count [49, 50].

The phase II REALIZE study evaluated the efficacy and safety of a new strategy of ruxolitinib dosing featuring a reduced starting dose and delayed dose escalation in

patients with MF-related anaemia. The primary endpoint was the proportion of patients achieving ≥50% reduction in spleen size at week 24. Secondary endpoints included anaemia and transfusion requirements, safety, and assessment of constitutional symptoms. The study included 51 MF patients with splenomegaly on physical examination and a haemoglobin level <10 g/dL. During the first 12 weeks, patients received 10 mg RUX twice daily, regardless of their baseline PLT count. In patients with stable PLT counts >100 G/L and without 50% reduction in spleen length, the dose was increased to 15 mg. In the absence of splenic response, the dose was increased by 5 mg every four weeks up to a maximum dose of 25 mg if the PLT count was >200 G/L. By weeks 24 and 48, 26.2% and 32.4% of patients, respectively, had received a total daily dose of RUX ≥30 mg. At study end, 12.0% of patients maintained the increased doses and 30.0% maintained the initial dose. The great majority of patients continuously receiving the initial dose achieved a splenic response at week 12 or later (11/15 patients). Median Hb levels remained stable throughout the study period, with no increase in transfusion requirements. Platelet counts and Hb levels were similar in patients who received an increased dose compared

to those in whom the dose had not increased during the study. The results of this study support the efficacy of lower doses of RUX in reducing spleen size and constitutional symptoms without significantly worsening anaemia. This dosing strategy may be appropriate for MF patients with clinically significant anaemia [50].

Fedratinib

The second JAK inhibitor approved for the treatment of MF patients was fedratinib (FED). This drug was approved in 2019 based on the results of the JAKARTA and JAKARTA-2 studies, which included patients with intermediate-2 or high-risk MF according to IPSS, previously untreated with a JAK2 inhibitor (JAKARTA) or resistant/intolerant to RUX (JAKARTA-2) [51, 52]. The primary endpoint in both studies was the percentage of patients with SVR35, and the secondary endpoint was TSS50. JAKARTA included 289 patients who were randomised to one of three arms: placebo, or FED at a dose of 400 mg, or FED at a dose of 500 mg. After 24 weeks of treatment, the primary endpoint was achieved by 1%, 36% and 40% of these patients, respectively. The reduction of general symptoms differed significantly between the placebo arm (7%) and both FED arms, although the higher dose did not translate into a higher percentage of patients meeting this endpoint (36% of patients receiving 400 mg and 34% of patients receiving 500 mg). Anaemia was a significant complication of treatment and was dose-dependent: grade 3 or higher occurred in 43% of patients receiving 400 mg and in 60% of patients receiving 500 mg, whereas in the placebo arm it occurred in only 25% of patients [51]. JAKARTA-2 was a single-arm phase II study evaluating the efficacy of FED at a dose of 400 mg in intermediate-2 and high-risk patients resistant to or intolerant of RUX [52]. The study included 97 patients, 30% of whom met the primary endpoint of SVR35, and TSS50 was achieved in 26% of patients by the end of cycle 6 [52]. Grade ≥ 3 anaemia occurred in 38% of patients and was the most common reason for dose reduction, apart from increased serum lipase activity. In 2013, clinical trials with FED were stopped due to a suspected case of Wernicke's encephalopathy (WE), which led to premature termination of the JAKARTA-2 study.

The phase IIIb FREEDOM study evaluated the efficacy and safety of FED in patients with intermediate- or high-risk MF and platelet counts ≥ 50 G/L previously treated with RUX [53]. The study protocol included clear guidelines for mitigating the risk of gastrointestinal AEs, thiamine supplementation, and close monitoring for signs of encephalopathy. These actions included delaying or modifying the FED dose, prophylactic, and supportive use of antiemetics (e.g. ondansetron), supportive use of antidiarrhoeal medications, and dietary modification. Due to the COVID-19 pandemic, only 38 patients were included in the study. In the

efficacy population ($n = 35$), nine patients achieved the primary endpoint of SVR35 at the end of cycle 6 (25.7%; 95% CI 12.5–43.3); and 22/38 (62.9%) patients showed the best overall response of SVR35 at the end of treatment. Sixteen (44.4%) patients achieved $\geq 50\%$ reduction in constitutional symptoms after six cycles of treatment. Haematological AEs occurred in 28 (73.7%) patients. Grade 3/4 anaemia and thrombocytopenia were the most common AEs, occurring in 15 (39.5%) and nine (23.7%) patients, respectively. Gastrointestinal AEs were reported in 34 (89.5%) patients, most commonly grade ≤ 2 . The most common gastrointestinal AE was constipation (50%). The incidence of diarrhoea was highest in cycle 1, and the incidence of vomiting generally decreased over time. There were no cases of grade ≥ 3 nausea, vomiting, or diarrhoea. Compared to the JAKARTA-2 study, the frequency of gastrointestinal AEs was lower, and no patient developed encephalopathy [53].

The FREEDOM2 study was a multicentre, open-label, randomised, controlled phase III study including patients with intermediate-2 or high-risk MF with known resistance or intolerance to RUX who received FED at a dose of 400 mg/d or BAT (randomisation 2:1) [54]. Patients received prophylactic antiemetics and thiamine supplementation, and symptomatic antidiarrhoeal medications as needed. The primary endpoint was a SVR35 rate after six months of treatment. The study included 201 patients (134 in the FED arm, 67 in the BAT arm), of whom 52 received RUX. The primary endpoint (SVR35 at six months) was achieved by 36% of patients receiving FED compared to 6% of patients receiving BAT (95% CI 20–39, $p < 0.0001$). Grade 3 or higher adverse events occurred in 40% of patients receiving FED and 12% of patients receiving BAT. The most common adverse events were anaemia (9% in each arm) and thrombocytopenia (12% in the FED arm and 3% in the BAT arm). Gastrointestinal AEs were more common in the fedratinib arm compared to the BAT arm, but were mostly grade 1/2 [54].

Fedratinib is a treatment option for patients with intolerance to or failure of RUX therapy, allowing a splenic response to be achieved in c.33% of patients, although there is no advantage over RUX in terms of haematological toxicity.

Momelotinib

Momelotinib, another JAK kinase inhibitor, inhibits not only JAK1 and JAK2, but also the activin A receptor type I (ACVR1), reducing hepcidin levels and increasing the availability of iron used for Hb production [55]. The drug was registered by the Food and Drug Administration (FDA) and the European Medicine Agency (EMA) in 2023 for MF patients with anaemia. Phase I/II studies assessing the safety of the drug had already showed its beneficial effect on Hb levels in

patients with MF-related anaemia [56]. Reduced anaemia was observed in 45% of patients, and 52% of patients with TD at baseline achieved transfusion independence within eight weeks. The phase III SIMPLIFY-1 study compared the efficacy of momelotinib to that of RUX in 432 patients with high-risk, intermediate-2, and symptomatic intermediate-1 MF [57]. SIMPLIFY-1 was designed as a non-inferiority study with the option to transfer to momelotinib after 24 weeks. SVR35, the primary endpoint of the study, was met by similar percentages of patients in both groups: 27% and 29% of patients treated with momelotinib and RUX, respectively ($p = 0.11$). The percentage of patients achieving TSS50, the secondary endpoint, was smaller in the momelotinib arm compared to patients treated with RUX (28% vs. 42%), which resulted in a failure of noninferiority criterion meeting. However, a beneficial effect of momelotinib on red blood cell parameters was observed in this study. Transfusion independence at 24 weeks was achieved by 67% of patients in the momelotinib group and 49% of patients in the RUX group ($p < 0.001$). The median number of RBC units transfused was also lower in the momelotinib group (0 vs. 0.4, $p < 0.001$). Grade 3/4 anaemia was observed in only 5.6% of patients treated with momelotinib, but in 23% in the RUX group. Peripheral neuropathy was observed in 10% of patients in the momelotinib group and 5% in the BAT group, although it was mild in most patients [57].

Another phase III study, SIMPLIFY-2, included 156 patients previously treated with RUX who required RBC transfusions, had grade 3/4 thrombocytopenia or anaemia during therapy, or required a dose reduction of RUX [58]. Patients received momelotinib or BAT (and 89% of patients in this group were pretreated with RUX). SVR35 was achieved by only 7% of patients in the momelotinib group and 6% in the BAT group. This low response rate was probably due to the initiation of momelotinib immediately after RUX discontinuation, without a washout period. TSS50 was reported in 26% of patients treated with momelotinib and 6% of patients in the BAT group ($p = 0.0006$). A beneficial effect of momelotinib on the red blood cell system was also observed in this study. Patients in the momelotinib group required fewer RBC transfusions (median 0.5 units/month vs. 1.2 units/month, $p = 0.39$) and a higher percentage of patients achieved TI (43% vs. 21%, $p = 0.0012$). The percentage of patients who developed grade 3/4 anaemia or thrombocytopenia was similar in both groups. Peripheral neuropathy was observed in 11% of patients in the momelotinib group, compared to zero patients in the BAT group [58].

MOMENTUM is a phase III study comparing the efficacy of momelotinib and danazol in 195 MF patients previously treated with a JAK inhibitor and with an Hb level < 10 g/dL [59]. In contrast to SIMPLIFY-1, a 2-week treatment-free period was required after discontinuation of JAK inhibitor therapy before initiation of momelotinib or danazol. As expected, both TSS50 (25% vs. 9%, $p = 0.0095$) and SVR35 at

24 weeks (23% vs. 3%, $p = 0.0006$) were reported more frequently in patients treated with momelotinib. Transfusion independence was achieved by 31% of patients in the momelotinib group and 20% of patients in the danazol group ($p = 0.0064$). The incidence of thrombocytopenia and neutropenia was similar in both groups [59].

Pacritinib

In a similar way to momelotinib, pacritinib inhibits ACVR1 in addition to JAK1 and JAK2 kinases, which translates into a beneficial effect on erythropoiesis. In addition, it has been proven that the drug inhibits IRAK1 (interleukin-1 receptor-associated kinase 1) [60]. The drug has been registered by the FDA for MF patients with a PLT count < 50 G/L. Unfortunately, pacritinib is not available in Europe due to a lack of EMA registration. The pivotal study of pacritinib, PERSIST-2, included 311 MF patients with a platelet count < 100 G/L [61]. Patients received pacritinib at a dose of 200 mg twice daily, or at a dose of 400 mg once daily, or BAT (sometimes RUX). In patients in the pacritinib groups, splenic and TSS50 responses were observed more frequently than in the BAT group. SVR35 was achieved by 18% vs. 3% of patients, respectively ($p < 0.00$) and 25% vs. 14% of patients, respectively ($p = 0.08$). An improvement in red blood cell parameters and a reduction in the number of RBC transfusions were also observed, especially in patients receiving pacritinib at a dose of 200 mg twice daily [61]. A retrospective analysis of pacritinib's efficacy in patients with platelet counts < 50 G/L showed similar efficacy and tolerability of the drug [62].

In the phase III PACIFICA study (NCT03165734), currently ongoing, pacritinib is being compared to treatment of physician's choice (*i.e.* low-dose RUX, danazol, steroids, HU) in patients with advanced MF and severe thrombocytopenia [63].

New drugs inhibiting activin receptor

Luspatercept is a recombinant fusion protein consisting of the extracellular domain of activin (ActRIIB) and the Fc fragment of IgG. It has the ability to bind to transforming growth factor $\beta 2$ (TGF- $\beta 2$) family ligands and to inhibit the activation of the ActRIIB receptor and signalling through the Smad2/3 pathway. Inhibition of the Smad2/3 pathway restores the maturation of erythroid precursors in the late stage of erythropoiesis [64]. Luspatercept is approved for the treatment of anaemia in patients with myelodysplastic syndromes (MDS) and β -thalassemia [65–67]. The results of clinical trials also indicate drug activity in MF patients. The phase II ACE-536-MF-001 study included 95 MF patients with anaemia, assigned to one of four cohorts:

- Cohort 1: TI patients, currently not treated with a JAK inhibitor;

- Cohort 2: TD patients, currently not treated with ruxolitinib;
- Cohort 3A: TI patients, currently receiving ruxolitinib;
- Cohort 3B: TD patients, currently receiving ruxolitinib [68].

The primary endpoint of the study was the response in terms of anaemia. In TI patients, the response was defined as an increase in Hb level ≥ 1.5 g/dL, and in TD patients as transfusion independence for at least 12 weeks. The highest efficacy of lusatercept was observed in cohort 3B (TD patients treated with RUX), with 26% of patients becoming transfusion independent. The primary endpoint was also met by 14% of patients in cohorts 1 and 3A, and by 10% of patients in cohort 2. Half of all TD patients had at least a 50% reduction in the number of transfusions [68]. The phase III INDEPENDENCE study is currently ongoing, evaluating the efficacy of lusatercept compared to a placebo in TD patients with MF treated with RUX (NCT04717414).

Sotatercept has a similar mechanism of action, binding to ligands of the TGF- β 2 family and inhibiting activation of ActRIIA receptor [69]. In a phase II study, the drug was used in MF patients with anaemia and an Hb level < 10 g/dL, in 27 patients as monotherapy, and in 19 in combination with RUX. A response in terms of improvement of red blood cell parameters was observed in 30% of patients in both groups [69].

Conclusions

Anaemia remains a significant problem in the treatment of patients with MF. The emergence of new therapeutic options gives rise to the hope that it will be possible to improve red blood cell parameters in a higher percentage of patients. Newer JAK inhibitors such as pacritinib and, above all, momelotinib, additionally improve red blood cell parameters in a significant percentage of patients thanks to an additional mechanism of action based on ACVR1 inhibition, while maintaining the characteristic ability of JAK inhibitors to reduce splenomegaly and general symptoms.

Great expectations await the results of studies with lusatercept and sotatercept, drugs with the ability to bind to ligands of the TGF- β 2 family as well as to inhibit the activation of the ActRII receptor and signal transduction through the Smad2/3 pathway, thereby restoring the maturation of erythroid precursors in the late stage of erythropoiesis.

Numerous new drugs with very different mechanisms of action are currently being tested in clinical trials. It is expected that their action will not be limited to reducing spleen size and general symptoms of the disease. The preliminary efficacy results of many of these drugs indicate an effect on reducing bone marrow fibrosis and the load of the mutant allele *JAK2*, *CALR*, *MPL*, as well as improving normal

haematopoiesis, including red blood cell parameters. The molecules in advanced clinical trials include:

- pelabresib — an inhibitor of BET (Bromodomain and Extra-terminal) proteins;
- navetemadin — an inhibitor of HDM2 (human double-minute homologue);
- imetelstat — a telomerase inhibitor;
- navitoclax — an inhibitor of BCL-2/BCL-xL (B-Cell Lymphoma-2/extra-large);
- bomedemstat — an inhibitor of LSD1 (lysine-specific demethylase 1).

These molecules are being tested both in first line treatment (as add-on therapy to RUX) and in patients with sub-optimal response or resistance to JAK inhibitors. Initial results of these studies prompt the hope that many of these new drugs will find a place in MF therapy, increasing the efficacy of JAK inhibitors and reducing their haematological toxicity [70, 71].

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Use of indocyanine green tracer in biopsy of lymph nodes in child with Nijmegen breakage syndrome

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Keywords: indocyanine green, ICG, lymphoproliferative disorder, Hodgkin's lymphoma, Nijmegen breakage syndrome

Nijmegen breakage syndrome (NBS) is clinically characterized by a high predisposition to lymphoid malignancies [1]. Lymph node biopsy is a standard diagnostic method where there is a suspicion of lymphoma. In recent years, fluorescence-guided techniques using tracers such as indocyanine green (ICG) have been introduced as an effective sentinel lymph node biopsy method to enhance tissue visualization. ICG imaging is a real-time method to evaluate organ perfusion based on direct visualization of the fluorescence emitted by ICG under near-infrared (NIR) light after injecting the tracer solution [2].

Improving intraoperative visualization of lymph nodes has become an important clinical challenge [3]. When pathological lymph nodes are deeply located in the abdomen or pelvis, blind dissection often leads to unnecessary invasiveness or a potentially increased risk of perioperative complications. Casaccia et al. and Gilbert et al. [4, 5] have reported laparoscopic biopsy as a method of choice. According to the literature, indocyanine green visualization has been established as a well-known tracer. In various oncological pathologies with secondary involvement of lymph nodes, ICG has already been established as a tracer of choice [6, 7]. The range of possible causes of abdominal and pelvic lymphadenopathy in the pediatric population is wide. Distinguishing between benign, atypical, and malignant lesions is crucial, especially in children with a genetic predisposition for malignancy. There is no consensus regarding the best diagnostic method of a minimally invasive way to obtain material for a histopathological examination in lymphoproliferative disorders in pediatric oncology.

This report aims to present the effectiveness of ICG-guided biopsy as an additional method of targeted surgical lymphatic tissue biopsy in pediatric patients.

The authors present the case of a 16-year-old girl with NBS and abdominal lymphadenopathy. In her medical history, bilateral surgical treatment of vesicoureteral reflux and right nephrectomy of the hypoplastic kidney had been performed. Screening ultrasound examination revealed enlarged lymph nodes in the hypogastric region. Magnetic resonance imaging (MRI) showed a pathological lymphatic mass spreading along the inferior vena cava and right iliac vessels. In addition to the high risk of lymphoproliferative disorders in NBS and the risk of postoperative adhesions (taking into consideration the right nephrectomy in the past), the patient was qualified for laparoscopic lymph node biopsy assisted with ICG fluorescence imaging. During the induction of general anesthesia, subcutaneous and intradermal injection of 2 mL of ICG solution in the bilateral inguinal region was performed. Intraoperatively, we detected clear and strong visualization of lymphatic tissue based on fluorescence. This allowed the surgeon to guide directly to the pathological lymph nodes (Fig. 1). Also, delayed ICG flow through the pathological lymphatic mass and heterogeneous enhancement patterns were observed compared to normal-looking other lymphatic tissue (according to the preoperative MRI). Pathomorphological examination of the material revealed Hodgkin's lymphoma (mixed-cellular subtype).

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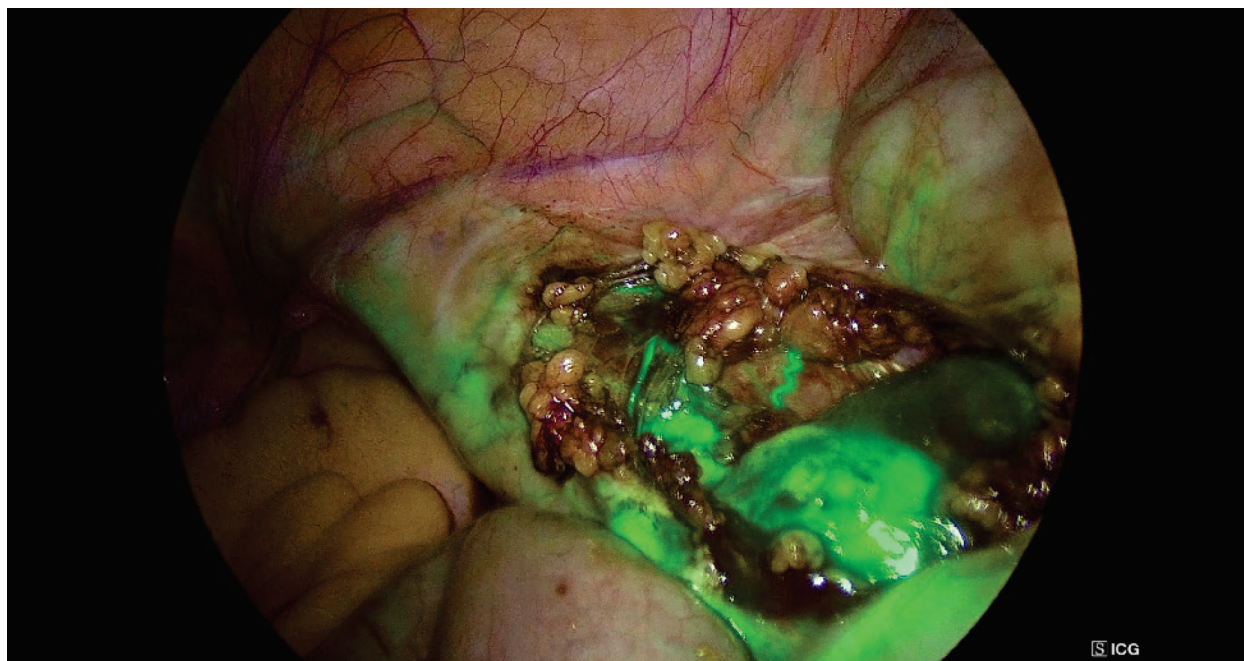


Figure 1. Right peri-iliac pathological lymphatic mass resected under fluorescence guidance

The postoperative course was uneventful, and the patient was transferred to the Department of Pediatric Oncology and Hematology three hours after the operation. She was qualified for chemotherapy.

Lymphomas need to be diagnosed by biopsy of lymphadenopathies with localizations that are often difficult to access, such as the retroperitoneum, neck, or mediastinum [8]. Initial reports of ICG application to identify pathological lymphatic tissue in lymphoproliferative diseases confirm that this is a feasible and safe diagnostic method. Excellent visualization of the local lymphatic system results in improved patient perioperative safety, a shorter operating time, and a reduced risk of both short- and long-term complications [6]. The pattern of fluorescence and tropism of ICG in pathological lymphatic mass is a promising area for further investigation. This intraoperative feature has been observed by Casaccia et al. [5], who reported a stop of the tracer flow in enlarged lymph nodes with a probability of obstruction of lymph drainage induced by pathological tissue.

Evaluation of ICG-guided biopsy of lymph nodes and its effect on the reduction of false negative results is an attractive option for pediatric patients. According to the FLABLY study, using ICG could simplify the lymph node search and help in the selection of a more accurate place for a biopsy [9]. More prospective studies with larger patient samples are needed to reach conclusions regarding the optimal method of use of fluorescent staining in lymphoproliferative disorders in the pediatric population.

Article information and declarations

Ethics statement

Authors declare that informed consent for publication was not obtained, as published data does not allow for patient identification.

Authors' contributions

All authors wrote, reviewed and agreed to the final version of the manuscript.

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Conflict of interest

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Acute promyelocytic leukemia in patient with chronic myeloid leukemia treated with tyrosine kinase inhibitors

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Keywords: acute promyelocytic leukemia, chronic myeloid leukemia, tyrosine kinase inhibitor, all-trans retinoic acid, arsenic trioxide, granulocyte alkaline phosphatase

Introduction

The authors report what is likely the first case of a patient with chronic myeloid leukemia (CML) who, after 11 years of treatment with tyrosine kinase inhibitors (imatinib and nilotinib), developed acute promyelocytic leukemia (APL) from a BCR::ABL-negative cell. Following the withdrawal of nilotinib and achieving remission of APL, the patient has remained in molecular remission of both diseases without further treatment for the past two years.

The occurrence of two or more neoplasms in the same patient is a rare phenomenon, especially when two uncommon cancers are present. This can occur due to several factors: coincidence, a treatment-induced malignancy, or both cancers arising from an underlying predisposition in the patient. The literature contains numerous reports of patients with two neoplasms, including cases of hematological malignancies. There are also reports of CML blast crisis manifesting as APL. However, the authors did not find any documented cases of APL resulting from a separate mutation outside of the CML clone [1–3]. In this report, the authors present such a case.

Case report

A 55-year-old male with hypertension and chronic gastritis presented in 2006 with a white blood cell count of 566 G/L, hemoglobin of 10.5 g/dL, and platelets of 300 G/L.

A peripheral blood smear revealed 23% blasts, and a bone marrow aspirate showed 15% myeloblasts, suggesting chronic myeloid leukemia (CML). Fluorescence in situ hybridization (FISH) testing confirmed the presence of the BCR::ABL fusion gene in 93% of cells, with a p210 transcript. The patient was pretreated with hydroxycarbamide (1 g/day), and then switched to imatinib (400 mg/day) despite the blast count exceeding 20%. This treatment decision was made by the previous clinical team, of which the current authors were not part. Therefore, the initial doses and therapy choice were outwith our control. Hematological remission and deep molecular response were achieved within six months.

In 2013, due to a loss of molecular response, the patient was switched to a second-generation tyrosine kinase inhibitor *i.e.* nilotinib (800 mg/day). A complete molecular response was achieved after six months. Subsequently, he maintained a deep molecular response (MR 4.5), with no evidence of translocation in karyotyping.

In 2021, 15 years into his treatment, the patient developed unexplained neutropenia and a reduced bone marrow reserve, confirmed by a hydrocortisone stimulation test. Bone marrow analysis revealed 20% blasts with morphological characteristics of acute promyelocytic leukemia (APL) and 24% blasts with phenotypic features of APL. Fluorescence in situ hybridization (FISH) testing confirmed the presence of the PML::RARA transcript, alongside molecular testing that detected the transcript, further confirming the diagnosis of APL. Treatment with all-trans retinoic acid

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(ATRA) and arsenic trioxide (ATO) was initiated, and despite complications caused by a severe SARS-CoV-2 infection, the patient achieved complete remission of APL.

Concurrently, CML remission remained at MR5. At the time of manuscript submission (two years after treatment cessation), the patient remains in remission for both CML (MR5) and APL (complete remission, minimal residual disease negative with no detectable PML::RARA transcript).

Discussion

There is no evidence in the literature suggesting that prolonged tyrosine kinase inhibitor (TKI) treatment induces secondary cancers, specifically acute promyelocytic leukemia [4]. The safety profiles of imatinib and nilotinib do not include any such warnings. Additionally, the chemical structure of these agents (imidazole derivatives) does not indicate mutagenic potential. Therefore, it is difficult to attribute this patient's APL development to prior CML treatment.

An intriguing question is whether a relationship exists between the two leukemias in this patient. Firstly, there is no indication that APL developed from a CML clone. At the time of symptomatic APL, 16 of 20 metaphase cells showed a t(15;17)(q24;q21) translocation, indicating the presence of the PML::RARA transcript (6.24×10^3 copies/5 μ L of cDNA) and very low levels of the BCR::ABL transcript (MR5). This suggests that APL arose from a separate cell population independent of the CML clone.

Is it possible that there is a connection between these two events, aside from mere coincidence? While such a relationship cannot be entirely ruled out, both translocations i.e. t(9;22) and t(15;17) occur during mitotic rosette formation, where chromosomes 9, 22, 15, and 17 are in close proximity, possibly facilitating these translocations. It is conceivable that a defect in this patient may have predisposed him to such translocations [5]. However, this single case does not provide conclusive evidence. Future reports of similar cases may allow for more detailed molecular investigations.

Acute promyelocytic leukemia (APL) is usually diagnosed as a *de novo* disease. However, there have been cases where APL has developed based on another malignancy or following prior cytotoxic treatment. These cases include patients with breast cancer, prostate cancer, testicular cancer, and pancreatic cancer. Among the drugs used in patients who developed secondary APL are alkylating agents (e.g. cyclophosphamide), topoisomerase II inhibitors (e.g. doxorubicin), and radiation therapy. The interval between treatment and the occurrence of APL typically ranges from four to 11 years [6–8].

Conclusions

Both chronic myeloid leukemia and acute promyelocytic leukemia can occur *de novo* or as secondary malignancies following prior hematological cancer treatments.

In this case, it is unlikely that APL was secondary to the patient's previous CML treatment. Currently, diagnostic tools are inadequate to definitively determine whether, and if so how, the two cancers in this patient are related.

Article information and declarations

Ethics statement

Authors obtained patient's informed consent for publication.

Authors' contributions

AB – manuscript preparation, data collection; NB-W – manuscript preparation; YK – data collection; WWJ – coordination, conceptualization, linguistic editing; KB-J – conceptualization, linguistic editing, final approval.

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Conflict of interest

The authors declare no conflict of interest.

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