Gdański Uniwersytet Medyczny

Rozprawa doktorska

Zależność pomiędzy dynamiką zmian molekularnych a obrazem klinicznym w nowotworach mieloproliferacyjnych

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Afiliacja

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Podziękowania

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SPIS TREŚCI

I. WYKAZ PRAC WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

1. Praca oryginalna

Autorzy: Patryk Sobieralski, Aleksandra Leszczyńska, Maria Bieniaszewska

tytuł artykułu: *Late polycythemic transformation in JAK2 - mutated essential thrombocythemia patients—characteristics along with a validation of 2016 WHO criteria*

Nazwa czasopisma, rok, doi: European Journal of Haematology, 2019, 10.1111/ejh.13320

IF 2,22| MNiSW 70 | Q3

2. Praca kazuistyczna

Autorzy: Patryk Sobieralski, Maria Bieniaszewska, Aleksandra Leszczyńska, Monika Żuk, Bartosz Wasąg, Jan Maciej Zaucha

tytuł artykułu: *Secondary chronic myeloid leukemia in a patient with CALR*

and ASXL1-mutated primary myelofibrosis

Nazwa czasopisma, rok, doi: International Journal of Hematology, 2022, 10.1007/s12185-022-03331-x

IF 2,1 | MNiSW 70 | Q4

3. Praca oryginalna

Autorzy: Maria Bieniaszewska, Patryk Sobieralski, Aleksandra Leszczyńska, Magdalena Dutka

tytuł artykułu: *Anagrelide in essential thrombocythemia: Efficacy and long-term consequences in young patient population*

Nazwa czasopisma, rok, doi: Leukemia Research, 2022, 10.1016/j.leukres.2022.106962

IF 2,7 | MNiSW 70| Q3

4. Praca oryginalna

Autorzy: Patryk Sobieralski, Bartosz Wasąg, Aleksandra Leszczyńska, Monika Żuk, Maria Bieniaszewska

tytuł artykułu: *The molecular profile in patients with polycythemia vera and essential thrombocythemia is dynamic and correlates with disease's phenotype*.

Nazwa czasopisma, rok, doi: Frontiers in Oncology, 2023, 10.3389/fonc.2023.1224590

IF 4,7 | MNiSW 100 | Q2

Łączna wartość wskaźnika oddziaływania (IF): **11,72**

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II. WYKAZ STOSOWANYCH SKRÓTÓW

MPN – myeloproliferative neoplasm (nowotwór mieloproliferacyjny)

ET – essential thrombocythemia (nadpłytkowość samoistna)

PV – polycythemia vera (czerwienica prawdziwa)

PMF – primary myelofibrosis (pierwotna mielofibroza)

CML – chronic myeloid leukemia (przewlekła białaczka szpikowa)

mPV – masked polycythemia vera (ukryta czerwienica prawdziwa)

VAF – variant allele frequency (obciążenie allelem zmutowanym)

NGS – next-generation sequencing (sekwencjonowanie następnej generacji)

HU – hydroxycarbamide (hydroksymocznik)

FISH – fluorescent in-situ hybridization (fluorescencyjna hybrydyzacja in situ)

RQ-PCR – real-time quantitative polymerase chain reaction (ilościowa łańcuchowa reakcja polimerazy)

Słowa kluczowe w języku polskim

Czerwienica prawdziwa; nadpłytkowość samoistna; sekwencjonowanie następnej generacji; profil molekularny

Słowa kluczowe w języku angielskim

Polycythemia vera; essential thrombocythemia; next-generation sequencing; molecular profile

III. STRESZCZENIE W JĘZYKU POLSKIM

Zależność pomiędzy dynamiką zmian molekularnych a obrazem klinicznym w nowotworach mieloproliferacyjnych.

Grupa nowotworów mieloproliferacyjnych (MPN) Philadelphia-ujemnych obejmuje trzy główne jednostki chorobowe – czerwienicę prawdziwą (PV), nadpłytkowość samoistną (ET) oraz pierwotną mielofibrozę (PMF) – charakteryzujące się nadprodukcją jednego lub wielu elementów morfotycznych krwi. Dla rozwoju MPN kluczowe jest występowanie mutacji w jednym z trzech genów – *JAK2, MPL* lub *CALR* – tzw. mutacji wiodących. W ostatnich latach zidentyfikowano również szereg mutacji towarzyszących, obejmujących inne geny odpowiedzialne za regulację hematopoezy, mogące występować równolegle do mutacji wiodących.

Badania przeprowadzone w innych schorzeniach układu krwiotwórczego wskazują, że w przypadku mutacji towarzyszących, nie tylko obecność, ale i obciążenie zmutowanym allelem wpływa na fenotyp choroby. W nowotworach mieloproliferacyjnych znaczenie mutacji towarzyszących jest dotąd nieznane. Wyjątek stanowi PMF, w której, zgodnie z najnowszymi systemami prognostycznymi, wykrycie mutacji towarzyszących określa grupę ryzyka. W przypadku PV i ET - schorzeń o powolnym przebiegu, gdzie potrzeba wielu lat na zainicjowanie choroby, wystąpienie objawowej manifestacji, rozwój powikłań lub progresji - badania oceniające wpływ zmian molekularnych są znacznie utrudnione.

Stworzenie bazy danych pacjentów z MPN leczonych w Klinice Hematologii i Transplantologii w Gdańsku było kluczowe dla rozpoczęcia badań nad tą grupą chorych. Przeprowadzona analiza danych klinicznych wykazała, że pomimo powolnego przebiegu, fenotyp MPN charakteryzuje się dynamiką. W badaniu analizującym fenomen transformacji policytemicznej ET skonfrontowano dane kliniczne ze zmieniającymi się kryteriami diagnostycznymi i ilościowym obciążeniem zmutowanym allelem mutacji wiodącej. Nie wykazano jednak jednoznacznego wyjaśnienia dla mechanizmów odpowiadających za transformację. Analiza danych cytogenetyczno – molekularnych kolejnej pacjentki, ze współistnieniem mielofibrozy i przewlekłej białaczki szpikowej, umożliwiła prześledzenie ewolucji klonalnej i ujawniła współwystępowanie zdarzeń genetycznych charakterystycznych dla odrębnych jednostek chorobowych. W rezultacie umożliwiło to wdrożenie właściwego leczenia. W kolejnej pracy poddano analizie wpływ zastosowanego leczenia na fenotyp choroby w grupie młodych pacjentów z ET i historią wieloletniego leczenia anagrelidem. Wykazano, że zarówno odpowiedź na leczenie jak i profil toksyczności różni się pomiędzy grupami pacjentów o różnym profilu molekularnym.

Wyniki powyższych badań jednoznacznie wskazywały na dynamiczny charakter fenotypu choroby u pacjentów z MPN. Wysunięto hipotezę, że to zmieniający się profil molekularny napędza mechanizmy odpowiedzialne za tę dynamikę. Jako ostateczną część pracy zaplanowano i przeprowadzono unikatowe badanie, w którym szczegółowo zbadano profil molekularny u pacjentów z rozpoznaniem PV i ET w algorytmie dynamicznym. Dzięki przeprowadzeniu analizy molekularnej w dwóch, odległych (mediana 104 miesiące) punktach czasowych, retrospektywnie wydłużono czas obserwacji. Za

pomocą sekwencjonowania następnej generacji, przeprowadzono szczegółową analizę sekwencji 37 genów odpowiedzialnych za regulację hematopoezy. Dzięki porównaniu występowania poszczególnych mutacji oraz ilościowego obciążenia zmutowanym allelem w dwóch punktach czasowych, potwierdzono dynamiczną naturę profilu molekularnego u pacjentów z PV i ET. Ujawniono zależności pomiędzy występowaniem określonych wariantów i rozwojem wtórnego włóknienia szpiku, powikłań zakrzepowo-zatorowych oraz odpowiedzią na leczenie. Ponadto, skonfrontowano znaleziska z istniejącymi systemami prognostycznymi, podkreślając ich ograniczoną użyteczność w świetle nowych doniesień.

Opublikowane wyniki badań nad charakterystyką pacjentów z PV i ET wskazują na użyteczność powtarzanych badań molekularnych w tej grupie chorych. Podkreślamy potrzebę dalszych badań w omawianym zakresie, mających na celu zrozumienie patogenezy choroby, ulepszenie modeli prognostycznych oraz rozwój nowych opcji terapeutycznych.

IV. STRESZCZENIE W JĘZYKU ANGIELSKIM

Dependency between the molecular profile and clinical picture in myeloproliferative neoplasms.

The group of Philadelphia chromosome negative myeloproliferative neoplasms (MPNs) encompasses, among others, three classic entities – polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). These diseases are characterized by uncontrolled proliferation of myeloid progenitors, resulting in different phenotype depending on the most affected lineage. Specific molecular lesions have been identified to play pivotal role in the development of those diseases. Mutations in one of the three genes – *JAK2, MPL* or *CALR* – which are essential for the development of MPN's, are called *driver mutations*. Recently however, number of additional molecular aberrations accompanying driver mutations have been discovered. Those mutations are called passenger mutations and include numerous genes involved with hematopoiesis.

Results from the research conducted in other hematologic malignancies allows to hypothesize that both the occurrence and allelic burden of passenger mutations have an impact on the disease's clinical course. The spectrum of MPNs is an area yet under-researched, except for PMF in which the occurrence of some passenger mutations has been included in prognostic systems. The chronic nature of MPNs and the fact that the time needed for the disease to fully develop takes many years, severely hinders the molecular research in those diseases.

Designing and creating the clinical database of patients with MPNs treated in Hematology and Transplantology Department in Gdańsk allowed to conduct the research within this spectrum. By investigating groups of patients with PV and ET, we show that the disease phenotype in patients with MPNs is dynamic. We analyze the phenomenon of polycythemic transformation of ET, confront it with diagnostic criteria and allelic burden of driver mutation, however the mechanisms of transformation remain unclear. Next, we investigate a case of a patient who, after being diagnosed with ET for many years, first develops myelofibrosis and then presents with disease transformation to chronic myeloid leukemia. By precise cytogenetic and molecular analyses, we follow the clonal evolution in this patient, revealing the coexistence of two distinct entities, which finally allowed us to use successful, combined treatment. In another work we present the outcomes and adverse events in young patients with ET who were exposed to anagrelide for over ten years. We revealed that both the response to treatment and toxicity profile is different among various molecular subgroups of patients, underlying the need for careful implementation of this therapy in young patient population.

Taken together these studies confirm heterogenous nature of MPNs. We hypothesize that the mechanisms responsible for such diversity reside in the molecular profile. We designed and conducted the final study in which we extensively analyze molecular profile of patients with PV and ET in a unique, dynamic fashion. We retrospectively extended observation period by a median of 104 months by performing molecular analysis on archival samples from the diagnosis and comparing it with the corresponding follow-up samples taken from study participants. With the use of next-generation sequencing we perform molecular analyses of 37 myeloid-related genes. By analyzing the change in variant allele frequencies and revealing the acquisition of new mutations during the

disease, we confirmed the dynamic nature of the molecular profile of patients with PV and ET. We found connections between specific variants with the development of secondary myelofibrosis, thrombotic events, and response to treatment. We confronted our results with existing conventional and mutation-enhanced prognostic systems, showing the limited utility of available prognostic tools.

The results of this study underline the significance of repeated molecular testing in patients with PV and ET and indicate the need for further research within this field to better understand the disease, improve prognostic tools and broaden available treatment options.

V. WPROWADZENIE

Grupa nowotworów mieloproliferacyjnych Philadelphia-ujemnych (Ph- MPN), obejmuje trzy klasyczne jednostki chorobowe - czerwienicę prawdziwą (PV), nadpłytkowość samoistną (ET) i pierwotną mielofibrozę (PMF). W etiopatogenezie MPN kluczową rolę odgrywa obecność mutacji w jednym z trzech genów – *JAK2, CALR* lub *MPL*. Należą one do mutacji wiodących, powodujących permanentną aktywację szlaku sygnałowego JAK-STAT i ciągłe, niekontrolowane przekazywanie sygnałów proproliferacyjnych do jądra komórkowego, skutkujące nadprodukcją jednego lub wielu elementów morfotycznych krwi. Dzięki postępowi jaki dokonał się w zakresie diagnostyki molekularnej oraz zwiększającej się dostępności do nowoczesnych metod diagnostycznych, udowodniono, że oprócz mutacji wiodących, u pacjentów z MPN występują również mutacje towarzyszące, obejmujące szereg genów odpowiedzialnych za regulację procesu hematopoezy.

Mutacje towarzyszące stanowią obiekt zainteresowań w kręgu chorób hematologicznych. Rezultaty badań wskazują, że występowanie mutacji towarzyszących w istotny sposób może wpływać na przebieg choroby i rokowanie pacjentów. W grupie MPN Ph(-), jedynie w przypadku PMF, charakteryzującej się najbardziej dynamicznym przebiegiem i niekorzystnym rokowaniem, wykazano prognostyczne znaczenie mutacji towarzyszących. Niewiele jednak wiadomo o występowaniu i roli tych mutacji w pozostałych dwóch MPN.

Zaplanowaną pracę badawczą rozpoczęto od stworzenia bazy pacjentów z rozpoznaniem Ph(-) MPN będących pod opieką Kliniki Hematologii i Transplantologii UCK. Dzięki uzyskanym danym, możliwe stało się dokładne prześledzenie losów, identyfikacja oraz analiza grup pacjentów, u których obserwowano dynamiczny przebieg choroby. Na podstawie uzyskanych danych przeprowadzono badania nad fenomenem transformacji policytemicznej w ET oraz skutkami wieloletniego leczenia anagrelide w populacji młodych pacjentów z ET. Ponadto, zgromadzone dane pozwoliły na identyfikację pacjentki, u której za pomocą szczegółowej analizy molekularnej scharakteryzowano niezwykle rzadkie zjawisko współwystępowania dwóch MPN. Przeprowadzone badania ugruntowały pogląd, że pomimo pozornie zbliżonego obrazu parametrów krwi obwodowej i wyjściowej prezentacji klinicznej, pacjenci z MPN stanowią grupę zróżnicowaną pod względem odpowiedzi na leczenie, występowania powikłań zakrzepowo-zatorowych, częstości progresji choroby do wtórnego włóknienia szpiku, akceleracji choroby do ostrej białaczki lub transformacji w inną jednostkę chorobową. Wysunięto hipotezę, że obecność mutacji towarzyszących oraz ich dynamika, czyli zarówno ilościowa jak i jakościowa zmiana w czasie trwania choroby, jest odpowiedzialna za heterogenny przebieg kliniczny u poszczególnych pacjentów. Fakt, iż ET i PV są chorobami przewlekłymi - do których rozwoju, wystąpienia powikłań oraz progresji potrzeba wielu lat - znacznie utrudnia prowadzenie badań nad dynamiką obrazu klinicznego w tej grupie chorych. Większość przeprowadzonych dotychczas badań skupia się na pomiarach wykonanych tylko w jednym punkcie czasowym, np. w momencie diagnozy lub w czasie progresji choroby. Podejście to nie pozwala na scharakteryzowanie dynamiki profilu molekularnego, a tym samym na korelację poszczególnych zdarzeń genetycznych

z fenotypem choroby. Ostatnią i główną część pracy doktorskiej stanowiło badanie profilu molekularnego u pacjentów z ET i PV w dwóch oddalonych od siebie punktach czasowych. Dzięki unikalnej konstrukcji badania, możliwe stało się potwierdzenie hipotezy o zależności dynamiki zmian molekularnych i obrazu klinicznego w nowotworach mieloproliferacyjnych.

VI. CELE PRACY

Cel zasadniczy:

- Potwierdzenie dynamicznego charakteru profilu molekularnego oraz jego wpływu na obraz kliniczny u pacjentów z rozpoznaniem czerwienicy prawdziwej i nadpłytkowości samoistnej

Cele dodatkowe:

- Konfrontacja użyteczności kryteriów diagnostycznych z przebiegiem klinicznym choroby pacjentów z czerwienicą prawdziwą i nadpłytkowością samoistną
- Ocena przydatności klinicznej stosowanych systemów prognostycznych w czerwienicy prawdziwej i nadpłytkowości samoistnej
- Identyfikacja czynników mogących odpowiadać za ewolucję klonalną w nowotworach mieloproliferacyjnych

VII. OMÓWIENIE PUBLIKACJI WCHODZĄCYCH W SKŁAD ROZPRAWY DOKTORSKIEJ

Publikacja 1.

Najczęściej występującym zdarzeniem molekularnym u pacjentów z rozpoznaniem MPN jest mutacja *JAK2Val617Phe*. U części pacjentów z rozpoznaniem ET obciążonych tą mutacją, w czasie trwania choroby obserwuje się zmianę fenotypu do spełniającego kryteria rozpoznania PV – tzw. transformację policytemiczną. Poniższe badanie miało na celu zbadać częstość występowania powyższego fenomenu w grupie pacjentów z ET oraz identyfikację potencjalnych czynników sprawczych.

W badaniu tym przeanalizowano dane kliniczno-laboratoryjne z czasu diagnozy u 136 pacjentów z rozpoznaniem ET, u których stwierdzono obecność mutacji *JAK2Val617Phe*. Zbadano częstość występowania transformacji policytemicznej w medianie czasu obserwacji 9 lat. Skonfrontowano parametry laboratoryjne grupy badanej z kryteriami diagnostycznymi dla PV z 2008 i 2016 roku oraz dla maskowanej czerwienicy prawdziwej (mPV)[1–3]. Dodatkowo, u 9 pacjentów, u których rozpoznano transformację policytemiczną, przeprowadzono ocenę ilościową obciążenia zmutowanym allelem (variant allele frequency, VAF) *JAK2Val617Phe* w dwóch punktach czasowych – w momencie pierwszej identyfikacji zmian molekularnych oraz w momencie rozpoznania transformacji.

Konfrontacja parametrów laboratoryjnych z czasu diagnozy z nowszymi kryteriami diagnostycznymi poskutkowała zmianą wyjściowej diagnozy z ET na PV w 10% przypadków oraz na mPV w 9% przypadków. W grupie pozostałych pacjentów, którzy wyjściowo nie spełniali kryteriów rozpoznania dla PV i mPV, u 14 obserwowano stopniowe narastanie stężenia hemoglobiny, które ostatecznie pozwoliło na rozpoznanie jawnej czerwienicy prawdziwej. U pacjentów z transformacją, u których oceniano dynamikę VAF dla *JAK2Val617Phe*, nie potwierdzono przypuszczanego wzrostu VAF w toku trwania choroby. Analiza ta pokazuje, że pacjenci z rozpoznaniem ET obciążeni mutacją *JAK2Val617Phe* stanowią niejednorodną grupę chorych, u których możliwe jest wystąpienie zjawiska transformacji policytemicznej. Mechanizmy odpowiedzialne za ewolucję fenotypu choroby nie są poznane, nie zależą od zmiany VAF dla mutacji wiodącej i wymagają przeprowadzenia dalszych badań.

Sobieralski P, Leszczyńska A, Bieniaszewska M.

Late polycythemic transformation in JAK2-mutated essential thrombocythemia patients—characteristics along with a validation of 2016 WHO criteria.

Eur J Haematol. 2019;103(6):558-563. IF = 2,22

Publikacja 2.

O ile analiza bazy danych pacjentów z Ph(-) MPN pozwoliła na identyfikację kilkunastu pacjentów z transformacją policytemiczną, to fenomen ewolucji fenotypu prowadzącej do koegzystencji dwóch MPN dotyczył tylko jednego przypadku. W MPN transformacja z jednej jednostki chorobowej do drugiej jest zjawiskiem rzadkim, o niezbadanych dotąd mechanizmach. Na podstawie charakterystyki pacjentki z rozpoznaniem mielofibrozy pierwotnej, u której obserwowano ewolucję choroby do przewlekłej białaczki szpikowej, za pomocą szczegółowej diagnostyki molekularnej, poddano analizie mechanizmy prowadzące do transformacji.

U omawianej pacjentki wyjściowo postawiono rozpoznanie ET. Podczas rutynowej diagnostyki molekularnej nie wykryto mutacji *JAK2Val617Phe* oraz fuzji *BCR-ABL1*. Po 10 latach trwania choroby, z powodu nasilających się objawów ogólnych przeprowadzono reewaluację i postawiono rozpoznanie mielofibrozy. Badania molekularne wykazały obecność patogennych wariantów w genach *CALR* (typ 2 mutacji) oraz *ASXL1*. Z powodu narastającej leukocytozy oraz progresji splenomegalii pomimo leczenia ruxolitynibem, powtórzono badanie cytogenetyczne, które wykazało obecność t(9;22) w 3/20 analizowanych metafaz. Znalezisko zostało następnie potwierdzone badaniem FISH oraz badaniem RQ-PCR, wykazującymi już obecność fuzji *BCR-ABL1* w prawie wszystkich analizowanych komórkach. Dzięki dostępności materiału DNA z czasu ewaluacji mieliśmy możliwość wykonania analizy molekularnej z użyciem techniki NGS, która potwierdziła obecność wcześniej wykrywanych wariantów w genach *CALR* i *ASXL1*, oraz umożliwiła ocenę VAF tych mutacji – odpowiednio 92% i 36%. Biorąc pod uwagę fakt, że w prawie 100% komórek wykryto w tym czasie fuzję *BCR-ABL1*, udowodniliśmy współistnienie zdarzeń molekularnych charakterystycznych dla MF oraz CML w jednym klonie komórkowym. Pomimo redukcji leukocytozy nie osiągnięto eradykacji klonu komórkowego po zmianie leczenia na imatynib - w kontrolnych badaniach molekularnych nadal stwierdzano obecność *BCR-ABL1* w prawie wszystkich komórkach oraz w badaniu NGS potwierdzono obecność mutacji w genach *CALR* i *ASXL1,* odpowiednio z VAF 85% i 33%. Biorąc pod uwagę koegzystencję dwóch, odmiennych mechanizmów molekularnych, rozpoczęto leczenie za pomocą dazatynibu i ruxolitynibu. Terapia ta pozwoliła na eliminację wykrywanego transkryptu *BCR-ABL1* oraz redukcję VAF tylko dla mutacji w genie *CALR* (do 55%) natomiast nie dla *ASXL1* (36%), co sugeruje, iż omawiana transformacja zaszła w klonie *CALR+/ASXL1-*.

Powyższy przypadek ukazuje jak niejednorodną grupą pacjentów są chorzy z rozpoznaniem MPN. Dzięki przeprowadzeniu wnikliwej diagnostyki molekularnej możliwe było śledzenie sekwencji zdarzeń genetycznych co pozwoliło na zastosowanie właściwego leczenia – w tym allogenicznej transplantacji komórek krwiotwórczych - oraz uzyskanie remisji. Ponadto, dzięki przeprowadzonej diagnostyce, szczegółowo analizujemy potencjalne mechanizmy ewolucji klonalnej. Podkreślamy jak istotną rolę odgrywają zaawansowane metody diagnostyki molekularnej w opiece nad pacjentami z MPN.

Sobieralski P, Bieniaszewska M, Leszczyńska A, Żuk M, Wasąg B, Zaucha JM

Secondary chronic myeloid leukemia in a patient with CALR and ASXL1-mutated primary myelofibrosis.

Int J Hematol. 2022;116(3):442-445. *IF = 2,1*

Publikacja 3.

Kolejnym etapem badań była weryfikacja hipotezy czy dynamika fenotypu choroby może być zależna od zastosowanego leczenia. Z posiadanej bazy danych wyodrębniono populację młodych pacjentów z ET, którzy poddani byli długoletniej eskpozycji na działanie anagrelidu. Zgodnie z obowiązującymi zaleceniami anagrelid jest lekiem przeznaczonym dla pacjentów z ET, klasyfikowanych jako grupa ryzyka wysokiego, u których stwierdzono oporność na pierwszą linię terapii. Ze względu na to, że część pacjentów, u których stosowane jest leczenie cytoredykcyjne nie przekracza 60 roku życia, konieczne jest poznanie efektów przedłużonej ekspozycji na leki cytoredukcyjnie w tej grupie pacjentów. W niniejszej publikacji przedstawiono analizę 48 pacjentów z rozpoznaniem ET, u których czas eskpozycji na anagrelid wynosił co najmniej 10 lat.

Analizując grupę badaną wykazano, że lepszą odpowiedzą na anagrelid cechują się pacjenci, u których wykrywalna jest mutacja *JAK2Val617Phe,* w porównaniu do pacjentów z mutacją w genie *CALR, MPL*, lub u których nie wykrywa się żadnej mutacji. Jednym z najczęściej obserwowanych zdarzeń niepożądanych była postępująca niedokrwistość, obserwowana dopiero po 10 latach leczenia, w szczególności dotycząca pacjentów z mutacją w genie *CALR*. Wykazano, że w grupie pacjentów leczonych anagrelidem, niedokrwistość niezwiązana z włóknieniem szpiku wynika ze zmniejszonej wrażliwości na erytropoetynę. Ponadto, u siedmiu pacjentów zaobserwowano pojawienie się wcześniej niewystępującego włóknienia w szpiku.

Udowodniono, że pacjenci obciążeni mutacją *JAK2Val617Phe* cechują się najkorzystniejszym profilem toksyczności – najrzadziej obserwowano u nich niedokrwistość i w żadnym przypadku nie stanowiła ona o modyfikacji dawkowania leku. Ponadto, żaden z pacjentów z mutacją *JAK2Val617Phe* nie rozwinął wtórnego włóknienia szpiku. Zwracamy uwagę na zróżnicowaną odpowiedź na leczenie i profil działań niepożądanych u pacjentów o odmiennym profilu molekularnym. Ze względu na rozwój włóknienia szpiku oraz niedokrwistości niezwiązanej z włóknieniem szpiku u pacjentów bez mutacji *JAK2Val617Phe,* podkreślamy konieczność ostrożnego stosowania anagrelidu w tej grupie pacjentów. Powyższe badanie ukazuję rolę zmian molekularnych w planowaniu terapii u młodych pacjentów oraz podkreśla potrzebę szerszej diagnostyki molekularnej u pacjentów z MPN.

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Anagrelide in essential thrombocythemia: Efficacy and long-term consequences in young patient population.

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Publikacja 4.

Bazując na obserwacjach wynikających z przeprowadzonych wcześniej badań wykazano, że fenotyp pacjentów z MPN cechuje się dynamiką. Wysunięto hipotezę, że to właśnie profil molekularny i jego zmiany w czasie odpowiadają za przebieg choroby u pacjentów z MPN. Korzystając z stworzonego repozytorium, zidentyfikowano grupę pacjentów z wieloletnim przebiegiem MPN, u których dzięki biobankowaniu materiału genetycznego uzyskano dostęp do zabezpieczonego DNA z czasu diagnozy. Stworzyło to unikalne warunki do retrospektywnego wydłużenia czasu obserwacji o medianę aż 104 miesięcy. Do badania włączonych zostało 49 pacjentów z rozpoznaniem PV lub ET. Od uczestników pobrano próbki krwi obwodowej, z których wyizolowano DNA. W pobranym materiale oraz odpowiadających mu próbkach historycznych u każdego z pacjentów przeprowadzono analizę molekularną z użyciem NGS. Do sekwencjonowania użyto zestawu Archer VariantPlex Core Myeloid kit (ArcherDX), pozwalającego na rzetelną analizę 37 genów, w których udowodniono częste występowanie mutacji w chorobach mieloproliferacyjnych. Dodatkowo, metoda ta umożliwiła zdefiniowanie ilościowego odsetka komórek obciążonych mutacją (VAF).

Przeprowadzona analiza genetyczna ujawniła występowanie 78 wariantów w 37 analizowanych genach w grupie badanej. U jednego pacjenta wykryto współistnienie dwóch mutacji wiodących. U czterech pacjentów wykryto niekanoniczne mutacje wiodące w genach *JAK2 i MPL*. Nie wykryto niekanonicznych mutacji wiodących w genie *CALR* u żadnego z pacjentów. U trzech pacjentek z rozpoznaniem ET leczonych HU obserwowano obniżenie VAF dla mutacji wiodącej *JAK2Val617Phe* poniżej poziomu wykrywalności w drugiej próbce. U dwóch pacjentów, u których nie wykrywano żadnych mutacji wiodących, potwierdzono występowanie mutacji w genach towarzyszących. Najczęściej zmutowanymi genami były *TET2* (18 wykrytych wariantów) i *DNMT3*a (10 wykrytych wariantów). Mutacje *TET2* występowały z podobną częstością w pierwszej i drugiej próbce oraz mediana ich VAF oscylowała ok 50%, sugerując germinalne pochodzenie. Mutacje *DNMT3a* były rzadko wykrywane w pierwszej próbce, natomiast często w drugiej próbce. Mediana ich VAF oscylowała ok 9%, sugerując somatyczne pochodzenie. Ilość wykrywanych wariantów w drugiej próbce była porównywalna u pacjentów wymagających zmiany leczenia, doświadczających powikłań zakrzepowo zatorowych i rozwijających włóknienie szpiku, natomiast znacząco większa u pacjentów z dłuższym czasem obserwacji (powyżej mediany) oraz obciążonych mutacją w genach *TET2* lub *DNMT3a*. U pięciu pacjentów obserwowano pojawienie się patogennych mutacji w genie *ASXL1* w drugiej próbce, co korelowało z rozwojem mielodysplazji lub włóknienia szpiku.

Otrzymane wyniki zostały użyte do określenia ryzyka choroby za pomocą nowych systemów prognostycznych – MIPSS-PV i MIPSS-ET[4]. W grupie pacjentów z ET, wyniki analizy genetycznej z drugiej próbki pozwoliły na zmianę grupy ryzyka na wysokie w pięciu przypadkach. Nie obserwowano powyższych zależności używając systemu MIPSS-PV, pomimo, że u jednego z pacjentów wykryto patogenny wariant w genie *TP53*. Dodatkowo przeprowadzono analizę z użyciem zaproponowanego kalkulatora ryzyka cytogenetyczno-molekularnego dla pacjentów z MPN, nie potwierdzając jego użyteczności w identyfikacji pacjentów z ryzykiem progresji do wtórnego włóknienia szpiku[5].

Aby zbadać zależność pomiędzy dynamiką zmian molekularnych a zastosowanym leczeniem, przeanalizowano niezależnie grupy pacjentów otrzymujących HU w monoterapii poprzez cały okres obserwacji oraz pacjentów, u których nie było wdrożone leczenie cytoredukcyjnie. Pacjenci leczeni HU mieli niższą medianę VAF mutacji wiodących wykrywanych w drugiej próbce, ale znacznie częściej dochodziło u nich do pojawienia się patogennego wariantu w genie *ASXL1*. U pacjentów, u których nie wdrożono leczenia, obserwowano stabilny, niski VAF mutacji wiodącej na przestrzeni analizowanych próbek oraz nie stwierdzono żadnego patogennego wariantu w obrębie wykrytych mutacji towarzyszących.

U dziewięciu pacjentów doszło do włóknienia szpiku w okresie obserwacji. Pacjenci, u których stwierdzono włóknienie szpiku, wykazywali trend wyższego VAF dla mutacji wiodącej przy diagnozie oraz znacznie wyższego VAF w drugiej próbce. Pacjenci ci otrzymywali również wyższą punktację w skali MIPSS, bazując zarówno na analizie z pierwszej jak i drugiej próbki. U pacjentów rozwijających wtórne włóknienie szpiku wykryto warianty patogenne, wcześniej opisywane również u pacjentów z PMF, w części obecne już w pierwszej próbce, a w części pojawiające się dopiero w drugiej próbce.

U dwóch pacjentów, sklasyfikowanych jako grupa ryzyka niskiego, doszło do powikłań zakrzepowo zatorowych w czasie obserwacji pomimo, iż nadal pozostawali w grupie ryzyka niskiego w czasie wystąpienia powikłania. Analiza molekularna wykazała narastanie VAF mutacji *JAK2Val5617Phe* u jednego z nich oraz obecność patogennego wariantu genu *ZRSR2*, który opisywano u pacjentów rozwijających powikłania zakrzepowo-zatorowe, u drugiego.

Podsumowując, w grupie badanej wykryto szereg mutacji towarzyszących. Uzyskane dane z analizy molekularnej zostały zestawione z indywidualną charakterystyką każdego pacjenta. Analizując zmiany w ilości wykrywanych mutacji, jak i VAF tych mutacji, potwierdzono hipotezę o dynamicznym charakterze profilu molekularnego u pacjentów z PV i ET. Wykazano korelację występowania niektórych wariantów z ryzykiem rozwoju wtórnego włóknienia szpiku. Konfrontując znaleziska wynikające z analizy molekularnej z istniejącymi systemami prognostycznymi, podkreślamy ograniczoną przydatność powyższych narzędzi. Wyniki tego badania wskazują na istotność przeprowadzania szczegółowej analizy molekularnej więcej niż raz w ciągu trwania choroby u pacjentów z PV i ET.

Sobieralski P, Wasąg B, Leszczyńska A, Żuk M, Bieniaszewska M

The molecular profile in patients with polycythemia vera and essential thrombocythemia is dynamic and correlates with disease's phenotype

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VIII. PODSUMOWANIE CAŁOŚCI ROZPRAWY

Wyniki opublikowanych badań umożliwiły pogłębienie wiedzy na temat roli mutacji towarzyszących oraz identyfikację problemów diagnostyczno-terapeutycznych u pacjentów z PV oraz ET. Udowadniając dynamiczny charakter fenotypu nowotworów mieloproliferacyjnych, zwracamy uwagę na potrzebę rozszerzenia obowiązujących kryteriów diagnostycznych i systemów prognostycznych o szczegółowe analizy molekularne. Podkreślamy konieczność uważnej interpretacji wyników badań molekularnych, z włączeniem oceny ilościowej, oceny dynamiki oraz oceny patogenności poszczególnych wariantów. Biorąc pod uwagę trendy w leczeniu pacjentów z PV i ET – w szczególności włączanie leczenia cytoredukcyjnego w grupie młodych pacjentów oraz opieranie decyzji terapeutycznych na nieprecyzyjnych kryteriach – wskazujemy na konieczność kontynuacji badań w zakresie diagnostyki molekularnej oraz podkreślamy istotność wielokrotnej ewaluacji choroby w trakcie jej trwania.

Bazując na wynikach licznych badań prowadzonych w szeregu innych niż MPN chorób hematologicznych, staje się jasne, iż fenotyp choroby jest dyktowany i modulowany przede wszystkim poprzez sekwencje zdarzeń cytogenetyczno-molekularnych, a w dalszej kolejności poprzez surowe czynniki takie jak wiek czy powikłania zakrzepowo-zatorowe. Jesteśmy zaledwie na początku drogi do wprowadzenia badań molekularnych w rutynowej diagnostyce chorób onkologicznych, zwłaszcza w hematologii, zwłaszcza w przypadku nowotworów mieloproliferacyjnych. Zdajemy sobie sprawę, iż większa część wiedzy o zdarzeniach molekularno-cytogenetycznych pozostaje wciąż nieznana, włączając w to modyfikacje epigenetyczne, wpływ czynników transkrypcyjnych, oddziaływanie z mikrośrodowiskiem oraz parakrynny wpływ komórek nowotworowych na pozostałe komórki, co wskazuje na potrzebę dalszych badań w tym zakresie.

IX. PIŚMIENNICTWO

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X. PUBLIKACJE WCHODZĄCE W SKŁAD ROZPRAWY DOKTORSKIEJ

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Late polycythemic transformation in *JAK2*-mutated essential thrombocythemia patients—characteristics along with a validation of 2016 WHO criteria

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Correction added on 4 October 2019 after first online publication: the country name in the authors' affiliation and correspondence address has been corrected.

Abstract

Introduction and objectives: The most common mutation within the spectrum of myeloproliferative neoplasms (MPNs) is a mutation in Janus kinase 2 gene (*JAK2V617F*). It has been observed that, during a course of disease, transformation from *JAK2*-mutated essential thrombocythemia (ET) to overt polycythemia vera (PV) can occur. Primary objective of this study was to show the incidence of mentioned phenomenon.

Methods: In this study, we analyzed data of 136 patients diagnosed with *JAK2*-positive ET observed for a median time of 9 years. We examined blood count of each patient at the time of diagnosis and confronted it with 2008 and 2016 WHO criteria for PV and mPV. Additionally, we analyzed *JAK2V617F* allele burden in two separate time points among selected cases.

Results: Confrontation with new criteria resulted in change of diagnosis to PV and mPV in 10% and 9% cases, respectively. Within remaining patients, 14 showed increasing hemoglobin concentration over several months during late course of disease, resulting in change of diagnosis to overt PV. We did not find suggested increase in *JAK2* allele burden among transforming patients.

Conclusions: Phenotype transformation to polycythemia was proven to be possible within the group of *JAK2*-mutated ET; however, cause of this effect remains uncertain.

KEYWORDS

JAK2V617F, mPV, myeloproliferative neoplasms, polycythemic transformation

1 | INTRODUCTION

The spectrum of Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) comprises three classic hematopoietic diseases—essential thrombocythemia (ET), polycythemia vera (PV), and primary myelofibrosis (PMF). MPNs are characterized by specific genetic abnormalities in driver genes including *JAK2*, *CALR*, or *MPL*. These acquired mutations in hematopoietic stem cells are leading to uncontrolled proliferation of all myeloid progenitors, resulting in a phenotype adequate to the most affected lineage. PV is characterized by clonal proliferation of erythroid progenitors resulting in erythrocytosis often accompanied with leukocytosis and thrombocytosis with panmyeloid bone marrow hyperplasia and high risk of thrombosis. In ET, megakaryocytic lineage is the most affected one, resulting in thrombocythemia and increased risk in both thrombotic and hemorrhagic events. PMF is a trilineage monoclonal proliferation associated with bone marrow fibrosis and extramedullary hematopoiesis. Within aforementioned mutations, *JAK2V617F* is the most common one and occurs in nearly 95% patients with PV and over 50% patients with ET and PMF.[1]

Unfortunately, distinction between aforementioned diseases is not always clear. It has been reported that small percentage of patients initially diagnosed with *JAK2*-mutated ET, within the course of disease

presented a phenotype change to clinically overt PV.[2] Those patients may represent an early stage of PV at the diagnosis, which has been referred to as masked polycythemia vera (mPV) or a complete phenotype transformation associated with a more complex mechanism, like loss of heterozygosity in *JAK2*-mutated allele. Several studies show that *JAK2*-mutated ET resembles PV in hematological presentation, that is, higher hemoglobin concentration, hematocrit value, and leukocyte count than in patients harboring *CALR* or *MPL* mutation.[3] Evolution from previously diagnosed ET to clinically overt PV is, because not being treated properly, associated with poorer outcome—higher risk of thrombotic events, shorter time of progression to myelofibrosis or transformation to acute leukemia.[4,5] The issue is that we currently do not have sufficient tools to extract patients who may transform to overt PV during the course of disease, which results in possible undertreatment and clinical complications, as mentioned above.

The aim of this study was to highlight the problem with clinical distinction between true ET and an early stage of PV within the spectrum of *JAK2V617F*-mutated patients and analysis of the phenomenon of late transformation of *JAK2*-mutated ET to clinically overt PV observed in the study group.

2 | MATERIALS AND METHODS

2.1 | Patients

The study group consists of 136 patients extracted from clinical database coded under D75.2 ICD-10. Patients diagnosed with essential thrombocythemia with confirmed *JAK2V617F*-positive mutational status have been selected and consecutively included in the study. The diagnosis of ET before 2008 was based on Polycythemia Vera Study Group criteria[6]; then, the diagnosis was verified according to WHO criteria.[7,8] Masked polycythemia vera (mPV) has been diagnosed according to criteria provided by Barbui et al.[9] We gathered data containing hemoglobin, hematocrit, platelet, and white blood cell count at the time of diagnosis and compared them with most recent values. Additionally, serum erythropoietin levels at any time from diagnosis were included, if available. Among selected patients that showed late transformation to PV, more complex data have been collected. This included type of treatment, hemoglobin concentration (Hb), and platelets count (PLT) at the time of transformation and circumstances when it occurred. Additionally, quantitative *JAK2V617F* analysis has been performed in selected cases. First measurement was taken using isolated DNA samples obtained in 2009. The second one was performed in the samples taken in the time of transformation. All patients have been regularly followed in the same hematological center.

2.2 | Methods

2.2.1 | JAK2 V617F analysis

Total genomic DNA was extracted from peripheral blood samples using a QIAamp DNA mini kit (Qiagen, Germany) according to the manufacturer's instructions.

At the stage of diagnosis, status of JAK2V617F mutation was examined using ARMS-PCR method described by Jones at al.[10]

JAK2V617F quantitative analysis was performed using JAK2 MutaQuant Kit (Qiagen, Germany) based on qPCR technique with hydrolysis probes. Results were expressed as ratio of JAK2V617F copies to total JAK2 copies (the sum of wild type and mutant allele) and given in percentage.

3 | RESULTS

Diagnosis of ET was based on PVSG criteria in 50 patients, 64 patients were diagnosed according to 2008 WHO criteria, and 22 according to revised 2016 WHO criteria. Hemoglobin concentration and platelets count of 136 analyzed patients met criteria for ET diagnosis, mentioned above. Characteristics of study group including Hb and PLT at the time of diagnosis and on the follow-up with addition of serum erythropoietin levels are shown in Table 1. Bone marrow biopsy prior to 2016 was performed only in uncertain cases, and complete data of marrow histopathology are unavailable.

When 2008 WHO criteria were used retrospectively to analyze patients diagnosed with PVSG criteria, 2 patients, both females, met hematological thresholds for PV, presenting with Hb 17.2 g/dL and 16.9 g/dL, respectively. If 2016 criteria were used to the whole group, 7 more patients met hematological criteria for Hb (range 16.4-17.3 g/dL) and additional 4 for Hct only (range 48.8%-51.2%). These 13 cases presenting polycythemic phenotype from the beginning were excluded from further analysis.

Using hematological criteria for mPV, that is, Hb values of 16.0-18.4 and 15.0-16.4 g/dL in men and women, respectively, we classified 23 out of 136 patients (17%) as mPV. After exclusion of aforementioned 11 cases meeting 2016 criteria for PV, we were still able to classify 12 patients out of remaining 123 (10%) as mPV, using Hb thresholds of 16-16.4 g/dL and 15-15.9 g/dL for men and women, respectively.

Finally, after aforementioned cases have been excluded, a total of 111 patients remained diagnosed as true ET. Inclusion and exclusion process is shown in Figure 1. Serum erythropoietin (EPO) has been measured in 70 (63%) of these patients. Values ranged from 0.6 to 176 IU/L (median 6.3 IU/L), and subnormal levels (<5 IU/L) have been found in 28 (25%) cases (median 3.4 IU/L). Among patients with subnormal EPO, Hb values at the time of diagnosis were ranging from 13.1 to 16.3 g/dL (median 14.5 g/dL).

In 14 cases, transformation to PV within late observation period was observed (Table 2). After median time of 15 years from diagnosis (range 3-21), patients presented with gradual increase of hemoglobin concentration and met hematological criteria for PV. The overall incidence rate was 1.02% patients per year of follow-up. In this group, change of clinical picture occurred during treatment with anagrelide (ANG)—7 patients and hydroxyurea (HU)—3 patients. Two patients developed clinical features of PV in the period without any treatment, after several years of stable remission achieved as a result of HU and ANG treatment. Four patients treated with ANG

TABLE 1 General characteristics of study group

n (%)	Total	F	M
Diagnosis	**136**	**92 (68)**	**44 (32)**
Age at diagnosis, years, mean (range)	51 (25-89)	52 (25-88)	50 (28-89)
Hb, g/dL (range)	14.36 (11.1-17.3)	14.09 (11.7-17.2)	14.94 (11.1-17.3)
PLT, G/L (range)	952 (436-1970)	946 (479-1970)	965 (436-1621)
WBC, G/L (range)	10 (5-22)	9.5 (5.2-20.5)	10.6 (5-22)
LDH, IU/L (range)	253 (129-681)	231 (136-671)	278 (129-681)
Follow-up Time, years, median (range)	9 (1-27)		
Hb, g/dL (range)	13.8 (8.1-18.1)	13.6 (9.6-18.1)	14.1 (8.1-17.2)
PLT, G/L (range)	441 (184-1094)	454 (184-1094)	414 (226-1046)
WBC, G/L (range)	7.8 (2.2-43)	7.5 (3.2-19.3)	8.3 (2.2-43)
EPO, IU/L (range)	10.2 (0.6-176)	11.4 (0.6-176)	7.5 (1.1-30)

transformed to PV after relatively long period (>10 years) of therapy. In three patients treated with HU, an increase in Hb occurred in association with stable, normal PLT count. In one case that became intolerant to HU after 18 years of effective treatment, transformation has been observed within 3 months after switching to ANG. At the time of diagnosis, Hb or Hct values did not suggest polycythemia in any of those 14 cases. Comparison of blood cell count and EPO levels between patients with and without transformation is shown in Table 3. Additionally, among four (30%) patients thrombotic events have been reported, including Budd-Chiari syndrome at the time of diagnosis (one patient), pulmonary thromboembolism in peri-transformation period (one patient), thrombosis of subclavian artery 7 years after transformation (one patient), and one patient suffering from pregnancy morbidity.

JAK2V617F allele burden quantification has been performed in nine patients with transformation (Table 4) in two separate time points. Average time between measurements was 7.4 years. Increase over 50% has been found in only one case. Other patients did not reach aforementioned threshold after transformation. Moreover, in 3 cases a decrease in allele burden has been observed. Also, type of treatment has been included for comparison but no regularity has been observed.

4 | DISCUSSION

Recently, there have been a few studies covering the issue of *JAK2*-mutated ET and its resemblance to PV.[9,11-13] These studies show that this group is inhomogeneous and comprises patients that initially

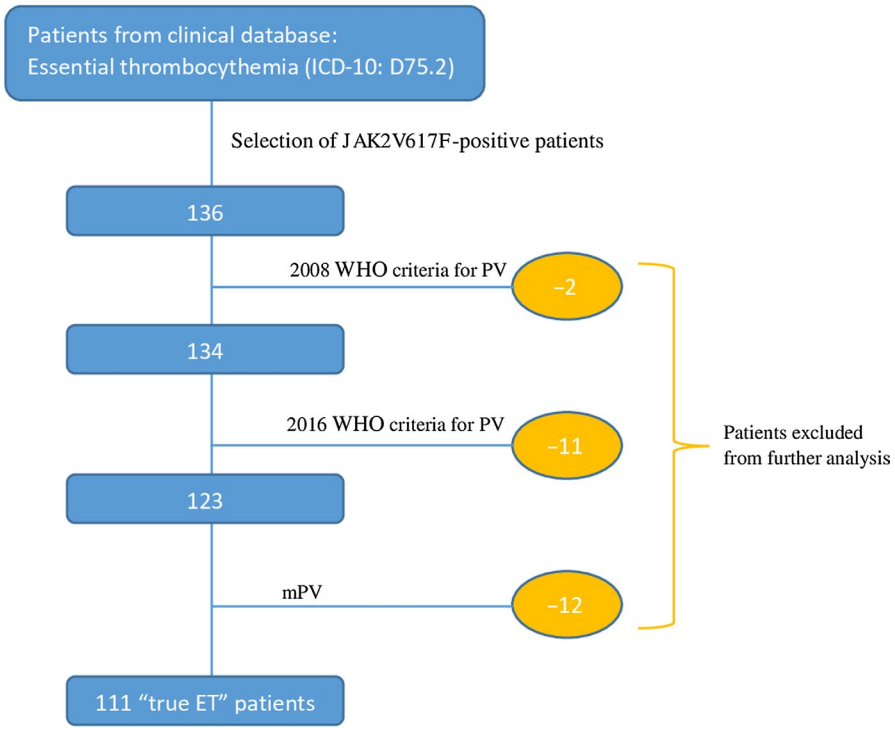

FIGURE 1 Inclusion criteria for study group

TABLE 2 Characteristics of patients with transformation

Therapy	n 14	Time of treatment years, median (range)	Time to transformation, months, median (range)	Hb at diagnosis, g/dL, median	Hb at transformation, g/dL, median	PLT at diagnosis, g/L, median	Serum EPO IU/L, median
ANG	6	15 (7-20)	6 (4-11)	13.8	16.3	1172	3.8
HU	3	n/a	n/a	14	17	1020	2.8
HU/ANG	1	18	3	14	16.1	1200	3.5
Discontinued	4	12	6	14	16.1	990	4.25

present with higher Hb concentration than patients without this mutation, or develop increasing Hb at some point during the course of disease.[2,14] In 2014, Barbui et al analyzed sensitivity of diagnostic thresholds for PV, implying that portion of PV patients are underdiagnosed. They suggested the existence of an early stage of PV that mimics ET, but presents as clinically overt PV during further observation.[11] Following the hypothesis of Barbui et al that patients with Hb values of 16.0-18.4 g/dL and 15.0-16.4 g/dL in men and women, respectively, are in fact an early stage of PV rather than ET[9]; it indicates that fraction of patients is still underdiagnosed. This group has been referred to as masked polycythemia vera (mPV). The reported incidence before introduction of 2016 guidelines, varied from 15% to 35% of *JAK2*-positive ET, depending on used criteria.[13] In our study group, we found 23 (17%) patients meeting this criterion. Discussion around this topic contributed to redefining MPN diagnostic criteria in 2016 resulting in lowering the hematological criteria for PV, that is, Hb > 16.5 or > 16 g/dL in males and females, respectively.

Using 2008 and 2016 WHO criteria, we evaluated whether all patients diagnosed earlier as ET, meet new recognition conditions. We found that 13 (10%) patients at the time of diagnosis had Hb values above 2016 diagnostic thresholds for PV, which lines up with a suggestion that diagnostic criteria for Hb prior 2016 were too permissive, causing misdiagnosis in a portion of patients. However, even when using new criteria for PV, there is still significant number of patients (with Hb 16-16.4 and 15-15.9 g/dL for men and women, respectively) that could be still diagnosed as mPV. In our study, those patients constituted for 10% (12 cases) of the analyzed group. It has been proved that those patients are at higher risk of thrombotic events, has shorter median lifetime expectancy, which results mainly from undertreatment on its early stage, and thus should be treated more aggressively.[5,9,12,15,16]

TABLE 3 Comparison between transforming and non-transforming patients

Patients	Without transformation	Transformation
N	97	14
Hb at diagnosis, g/dL, median (range)	14.1 (11.1-15.9)	14 (13.1-15.8)
PLT at diagnosis, G/L, median (range)	861 (451-1970)	1053 (680-1754)
EPO, IU/L, median (range)	7.9 (2.1-176)	3.4 (0.6-11)

Changes in 2016 WHO criteria for PV also include promoting bone marrow histopathology from a minor to a major criterion. This change renders it necessary for diagnosis, apart from cases harboring JAK-2 mutation with hemoglobin levels >18.5 g/dL in men (hematocrit, 55.5%) or >16.5 g/dL in women (hematocrit, 49.5%) and subnormal EPO level.[17] Unfortunately, since bone marrow biopsy was performed only in selected cases prior to 2016, we did not have the full data and were unable to compare findings from the time of diagnosis.

Even when using strict hematological criteria for ET and excluding patients that could be retrospectively diagnosed as mPV or PV, we still observed a phenotype change within remaining patients. The phenomenon of transformation from ET to PV has very little mentions in literature. Reported incidence of transformation in *JAK2*-mutated ET patients ranges from 1.4% to 29%, depending on analyzed study group.[2,3,14] The lowest incidence of 1.4% was found by Rotunno et al. In his study, 5 patients out of 369 transformed to PV, all of them harboring *JAK2* mutation.[3] However, in his report, time of follow-up was relatively short, with a median of 6 years (range 0.2-20). In another study, Barbui et al reported the incidence of transformation in 5% among 422 *JAK2*-mutated patients.[2] In this case, follow-up time was even shorter, with a median of 4.73 years. The highest rate of transformation was found by Rumi et al In their study, transformation occurred in 53 out of 466 patients (11%) all of them *JAK2*-positive.[14] They estimate the cumulative risk of evolution to PV for 29% at 15 years follow-up. That exceptionally high rate of conversion to PV has been questioned, suggesting that portion of these patients could be in fact diagnosed as mPV. In our study, after both patients who meet revised diagnostic criteria for PV and those with mPV has been excluded, we analyzed 111 patients in whom diagnosis of ET was undoubtful at the beginning of the disease. We found that among these patients, there were 14 cases (13%), which, during the late course of observation, presented a phenotype change from ET to PV. Median time of follow-up was 15 years (range 3-21), which is the longest observation period among very limited studies covering the issue of transformation. All of mentioned cases at the beginning had high platelet count combined with Hb and Hct values below diagnostic thresholds for PV not only according to 2016 WHO criteria, but also mPV, which justifies initial diagnosis of ET. This indicates that described change of phenotype was spontaneous and transforming patients form an entity separate from overt PV or mPV. Transformation within our group was suspected according to increase in Hb values. That

TABLE 4 *JAK2* allele burden quantification

N	JAK2V617F quantitive, %		Time from transformation, years	Former treatment
	2009	2017		
1	16.69	21.21	3	Discontinued
2	19.21	n/a	2	HU
3	22.18	23.04	6	ANG
4	32.06	26	4	ANG
5	36.82	60.44	5	HU/ANG
6	36.89	30.4	7	ANG
7	74.76	67.27	5	ANG
8	n/a	37.07	4	HU
9	n/a	47.44	7	ANG

particular group consisted of patients managed with different treatment methods, including those treated with ANG where rise in Hb concentration was highly unexpected. This indicates that treatment has no particular effect on transformation itself. Additionally, in 9 of these cases, quantification of *JAK2V617F* mutated cells has been measured. It has been suggested that *JAK2V617F* allele burden increases along with transformation from TE to PV. This effect was referred to as "loss of heterozygosity," that is, increasing allele burden over 50%.[2,18] In our study, we did not observe that trend. Our patients did not reach aforementioned threshold after transformation or even presented with a decrease in *JAK2V617F* mutated cells, except for one case. Similar results were reported by Rumi et al.[14] In their group, median mutant allele burden was 25.5% in patients who transformed to PV and 17.9% in those who did not. Additionally, type of treatment also did not appear to have any effect on allele burden among these patients.

An interesting finding was serum EPO levels in our group. Values ranged from 0.6 to 176 IU/L with a median value of 6.3 IU/L. Subnormal serum erythropoietin level (<5 IU/L) has been included as a minor criterion in 2016 revised WHO guidelines for PV diagnosis. The usefulness of this parameter has been discussed. Patients with PV have a tendency to present with lower values, but subnormal EPO has been reported also in up to 95% of patients with *JAK2*-mutated ET.[19] In our study group, we found much lower incidence of 24% only. However, subnormal serum EPO levels have been found in patients with transformation, range 0.6-11 IU/L, with median value of 3.4 IU/L. It has been reported that ET patients with subnormal EPO have tendency to transform to PV, are in greater risk of thrombotic events, but also are somehow protected from myelofibrotic transformation [19,20] According to this data, serum EPO level remains a supportive parameter during diagnostic process but may be an indicator for a transformation. This requires further analysis on a greater population of patients with a specified time of measurement.

Our study shows that in the group of *JAK2*-mutated ET there is a portion of patients that within the course of disease may transform to clinically overt PV. Cause of this transformation remains unknown, thus preventing us from effective screening. Additionally, increased sensitivity of new diagnostic hematological thresholds for PV has been noted; however, for proper diagnosis they should be used along with other parameters. Bone marrow histology from the time of diagnosis would have been an extremely useful finding, but as mentioned above, at that time it has not been routinely performed. This is another argument that supports the significance of early bone marrow examination, possibly in all *JAK2*-mutated ET patients.[21] Also, measurement of EPO levels might be helpful for diagnosis.[13] Patients from this group should be carefully monitored for signs of transformation and assessed properly when it occurs to prevent undertreatment and possible fatal complications.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

ETHICAL APPROVAL

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional research committee. Informed consent was obtained from all individual participants included in the study.

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CASE REPORT

Secondary chronic myeloid leukemia in a patient with CALR and ASXL1-mutated primary myelofibrosis

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Abstract

Development of secondary CML has only been casually described, with few reports attempting to analyze and explain the mechanisms behind this phenomenon. Reported cases vary with regard to presumed pathogenesis and clinical characteristics, but similarities can be observed. This report presents the case of a patient diagnosed with CALR and ASXL1-mutated primary myelofibrosis who developed CML 13 years after the initial diagnosis. In contrast with previously reported cases, this patient did not have JAK2 or ABL1 gene mutations, and also exhibited primary resistance to tyrosine kinase inhibitor (TKI) treatment. Here, we analyze the molecular evolution of CML and describe successful treatment with concomitant therapy including a TKI and JAK inhibitor. This report aims to deepen clinical experience and further broaden knowledge about chronic myeloproliferative neoplasms.

Keywords Chronic myeloid leukemia · Primary myelofibrosis · BCR–ABL1 · CALR · ASXL1

The acquisition of Philadelphia chromosome or *BCR–ABL1* gene rearrangement is unique for the development of chronic myeloid leukemia (CML). In case of suspicion of other chronic myeloproliferative neoplasms (MPN), initial exclusion of the *BCR–ABL1* fusion is mandatory. There have been only few reports of patients initially diagnosed with Philadelphia-negative (Ph−) MPN, who had documented evolution to clinically and molecularly overt CML. Here we report a patient diagnosed with primary myelofibrosis who developed Ph+ CML 13 years after the initial diagnosis that was resistant to tyrosine kinase inhibitors (TKI). The coexistence of *CALR* and *ASXL1* mutations and Philadelphia chromosome in a single subclone has not been reported as yet.

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A 53-year-old female patient was referred to our Department in September 2007 due to thrombocytosis. Platelet number of 952 G/l and significantly elevated LDH activity were the only laboratory abnormalities. She had no general symptoms and no palpable spleen. Routine workup included karyotype evaluation, which failed due to limited cell mitotic activity, and molecular tests for the presence of *BCR–ABL1* fusion gene and *JAK2V617F* mutation that were negative. Trephine biopsy showed megakaryocyte proliferation and atypia, along with correct age-adjusted cellularity (Table 1) Initially, the findings led to the diagnosis of essential thrombocythemia (ET). The patient was started with acetylsalicylic acid only since she was in the low-risk group. However, cytoreductive therapy with hydroxyurea was added shortly after due to aggravating general symptoms. Within the next years, she required continuous adjustments in doses of hydroxyurea due to both toxicity and unsatisfying control of platelets. Due to increasing general symptoms and concomitant spleen size in 2013, another trephine biopsy was made to evaluate the diagnosis. Histopathology confirmed megakaryocytic atypia with the addition of grade 1 reticulin fibrosis, along with correct age-adjusted cellularity. The subsequent trephine performed 3 years later revealed progression to grade 3 reticulin and collagen fibrosis, leading to the diagnosis of secondary myelofibrosis (sMF). With

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Table 1 Bone marrow, peripheral blood and molecular tests throughout the course of the disease

	2007	JUN 2013	SEP 2016	JAN 2019	JAN 2020	MAY 2020
Bone marrow						
Cellularity	70%	75%	80%	40%	Hard to assess	Minimal
Megakaryocyte features	Increased atypia	Increased atypia	Increased atypia	Increased atypia	Increased small	Increased atypia
Fibrosis, grade	0	1	3	3 + Col	3 + Col	3 + Col
Blast	Single	5%	Single	Single	Single	Single
WBC, G/l	9.1	4.4	4.49	15.28	76.2	7.15
Molecular findings	*JAK2*(+) *BCR–ABL1*(−)	Not assessed	*CALR*(+) *ASXL*(+)	Not assessed	*BCR/ABL*1 133% *CALR* 92% *ASXL1* 36%	*BCR/ABL*1 136% *CALR* 85% *ASXL1* 35%
Cytogenetics	Failed	Not assessed	Failed	t(9;22) 3/20	t(9;22) 15/15	t(9;22) 17/20

a retrospective application of 2016 WHO guidelines, our patient should have been initially diagnosed with prefibrotic MF, which underlines the significance of initial bone marrow examination. In 2017, due to gradual escalation of general symptoms, re-examination was made, including a second karyotype analysis, which was again not evaluable due to lack of cell mitotic activity. However, molecular tests showed the presence of *CALR* type 2 (c.1154_1155insTTGTC) and *ASXL1* exon 14 (c.1934_1935insG) genetic variants. In January 2019, considering aggravation of symptoms and increasing spleen size reaching 34 cm in USG, she was qualified for ruxolitinib treatment. Following karyotype examination, which was successful this time, revealed t(9;22) in 3 out of 20 analyzed metaphases. Unfortunately, molecular evaluation of this finding was not performed. Within the first 8 months of the treatment with a combination of ruxolitinib and hydroxyurea, reduction of spleen size was achieved (22 cm in USG), but during the following 4 months, due to a rapid increase in WBC reaching 85 G/l and loss of spleen response (33 cm in USG), ruxolitinib was stopped in December 2019. Subsequent evaluation detected 95% *BCR–ABL1*-positive cells in FISH. RT-PCR revealed the presence of *BCR–ABL1* p210 transcript, variant b2a2. RQ-PCR showed 133% of *BCR–ABL1/ABL1* ratio (IS). In NGS panel, insertion of c.1154_1155insTTGTC (p.Lys385AsnfsTer47) in exon 9 of the *CALR* gene and duplication of c.1934dup (p.Gly646TrpfsTer12) in exon 14 of the *ASXL1* gene were detected with a variant allele frequency (VAF) of 92% and 36%, respectively. No *ABL1* gene variants were documented. Trephine biopsy showed trilineage proliferation with coexisting grade 3 reticulin fibrosis and was suggestive of disease transformation (Table 1). However, marrow cytology was rather corresponding to the chronic phase of CML. The patient started treatment with imatinib, achieving a rapid decrease in leukocytosis and alleviation of symptoms; however, spleen size remained intact. After 5 months, despite the significant decrease in leukocytosis to 7.15 G/l, there was no change in *BCR–ABL1/ABL1* ratio (136%) (Fig. 1), general symptoms reoccurred, and again NGS revealed both *CALR* and *ASXL1* variants, with VAF of 85% and 35%, respectively. The decision was made to reintroduce the treatment with ruxolitinib, based on the idea that increasing symptomatic burden results from the reoccurrence of the MF clone. After 1 month of combination therapy with JAK-inhibitor

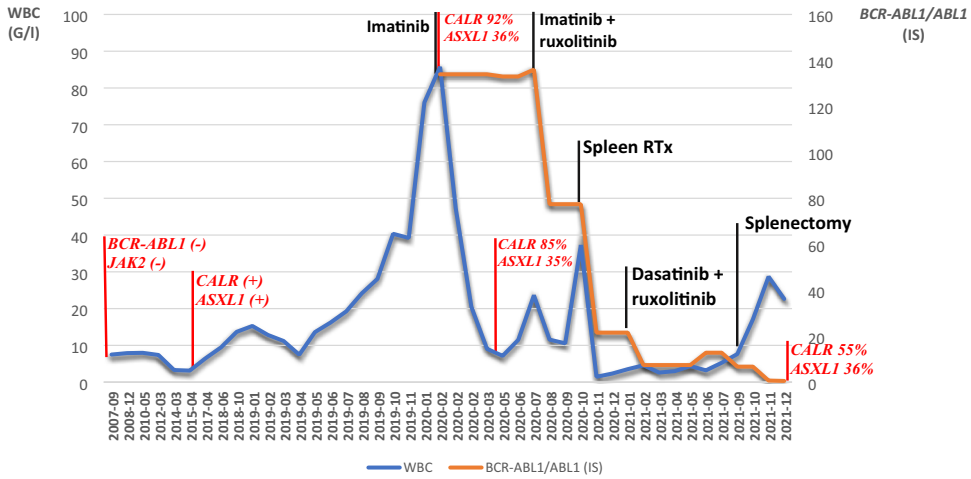

Fig. 1 White blood cell counts and BCR-ABL1/ABL1 ratios during the course of the disease

and tyrosine kinase inhibitor (TKI), the molecular evaluation showed a decrease in the *BCR–ABL1/ABL1* ratio to 77%, followed by a reduction to 21% at 3 months. The patient was referred and approved for allogeneic hematopoietic cell transplantation. Before the procedure, TKI was switched to dasatinib 50 mg QD due to still unsatisfying response, which allowed a further, stable decrease of *BCR–ABL1/ABL1* ratio. Matched unrelated donor has been found, and the patient began the preparation for the procedure, including spleen irradiation (8 Gy/8 fractions) because of persistent splenomegaly. The last molecular evaluation performed before the admission to the transplant unit showed a significant decrease of *BCR–ABL1/ABL1* ratio to **0.516%, along with a reduction in variant allele frequency for *CALR* but not *ASXL1* variant—55% and 36%, respectively.

The occurrence of CML as a secondary hematopoietic neoplasm is very rare and can be divided into three groups. First consists of cases in which a Ph+ clone was found during therapy of acute hematological malignancy and meets the definition of therapy related [1]. The second group includes patients with the diagnosis of Ph− MPN, in which putatively spontaneous transformation took place [2–9]. The third group includes patients with a history of autoimmune diseases or primary non-hematologic malignancies [10, 11]. The above classification is clinically relevant since the characteristics of secondary CML are different in each group. In therapy-induced transformation, an acquisition of p190 transcript is more prevalent and is associated with an evolution of already existing malignant clones. In contrast, in spontaneous transformation, almost all patients acquired p210 transcript, and the development of a new subclone is suggested, based on the fact that symptoms of primary disease reoccur after successful treatment of CML. In the last group, the characteristic of the disease is comparable to de novo CML. It is generally accepted that these are primary CML cases, which became more evident due to progress made in the survival of patients with solid tumors.

In the described case, at the point of highest leukocytosis and emergence of CML, both *ASXL1* and *CALR* variants were detectable at high VAFs. Combined treatment led to the reduction of *BCR–ABL1/ABL1* ratio and allele frequency for *CALR* variant, but not for *ASXL1*, suggesting that the transformation emerged from *CALR+/ASXL1−* clone. Preexisting Ph(−) MPN phase and acquisition of p210 *BCR–ABL1* transcript make this case most suitable for the second among the above-mentioned groups. Additionally, after suppression of *BCR–ABL1* kinase activity with TKI treatment, symptomatic burden characteristic for MF reoccurred, which supports the hypothesis of the phenotypic predominance of a more potent molecular alteration.

Homozygosity for type 2 *CALR* mutation is rare in patients with PMF [12, 13]. Our patient's karyotype showed neither trisomy nor deletion of chromosome 19, leaving uniparental disomy (UPD) as the only possible mechanism leading to homozygosity. However, a high allele burden for *CALR* mutations, persisting through successful TKI therapy, was reported in described cases of *CALR*-mutated MPNs with the transformation to CML [6, 7, 9]. Interestingly, in patients with *JAK2*-mutated MPNs, *JAK2* mutation was undetectable at the time of transformation but reemerged after successful TKI treatment. Chromosome instability caused by JAK–STAT overstimulation in patients with *JAK2*-mutated disease is suggested as a driving factor of clonal evolution [3]. Calreticulin malfunction resulting in independent STAT phosphorylation and proliferative advantage [13], with the addition of aberrant gene expression through *ASXL1* gene mutation, can equally justify the increased risk of acquiring additional chromosome abnormalities, including t(9;22). However, the characteristic of the response to TKI in the absence of *ABL1* gene mutations is in contrast with cases described in the literature. Despite allowing for a significant reduction in leukocytosis, imatinib showed almost no effect on the measured *BCR–ABL1/ABL1* ratio. This phenomenon could be explained if we assume that the malignant clone harbored both *CALR* variant and Ph-chromosome. Introducing a TKI decreased tyrosine kinase activity and limited extraordinary proliferative advantage achieved through *BCR–ABL1* fusion. However, due to *CALR* mutation coexistence, which promotes different proliferative advantage mechanisms, Ph+ cells, despite being inhibited could not be eliminated, translating into a persisting high *BCR–ABL1/ABL1* ratio. Hence, only the combination therapy with JAK-inhibitor and TKI allowed for a reduction of transcript levels. Another possibility is a kinase-independent activity in Ph+ cells [14], causing refractoriness to treatment and persistence of the disease, which is uncommon in CML but uninvestigated in patients with complex molecular architecture.

Another interesting finding is the initial good response to JAK-inhibitor treatment despite the presence of an already emerged CML clone, detected in 3 out of 20 metaphases. Ruxolitinib exerts its effects through non-selective JAK1 and JAK2 inhibition, resulting in both myelosuppression and anti-inflammation. Additionally, as a TKI, ruxolitinib is also effective in *JAK2*-nonmutated MPN clones. Hence, spleen size reduction in any MPN, not only JAK2-positive, is possible with ruxolitinib. Nonetheless, the positive effect on the splenic size and volume was temporary, suggesting dynamic changes in the clonal distribution.

The described case illustrates the molecular instability that occurs in patients with MPNs and suggests a distinct impact of each additional molecular abnormality on the existing clone which also translates to the clinical course. Thus, any surprising change in the clinical picture should stimulate the need for reevaluation of the initial diagnosis. We are aware that subsequently failed karyotype evaluations

throughout the MF phase of the disease is an important limitation of our report. However, at the time of initial diagnosis and later during the course of the disease the lack of mitotic activity for cytogenetics evaluation did not surprise us since it is a frequent complication in the diagnostic workup of patients with MF but not CML. Therefore, the first successful cytogenetic evaluation succeeded at the time of CML diagnosis indirectly spokes for the emergence of the CML clone at that time but not before. Nevertheless, we would like to emphasize the importance of pursuing a successful cytogenetics analysis.

Our findings may help in better understanding of clonal evolution of MPNs and facilitating treatment decisions in similar cases.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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Anagrelide in essential thrombocythemia: Efficacy and long-term consequences in young patient population

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ABSTRACT

According to the current treatment recommendations, anagrelide, an oral antiplatelet agent, is recommended as a second-line therapy for patients with high-risk essential thrombocythemia experiencing intolerance or refractoriness to first-line approach, such as hydroxyurea or pegylated interferon alpha-2a. If there is a need for introduction of cytoreductive treatment in young patients with a perspective of lifelong exposure, both the efficacy and long-term outcomes should be known. We present the analysis of 48 young patients, diagnosed with essential thrombocythemia below the age of 60, who were exposed to anagrelide treatment for over 10 years. Our observations show that the highest proportion of complete remissions without adverse events and disease progression is seen in the *JAK2*-mutated patients. By evaluating the changes in hemoglobin concentration and serum erythropoietin throughout the study, we were able to reveal the development of progressive anemia, resulting from diminished susceptibility to erythropoietin and unrelated to bone marrow fibrosis, in patients harboring *CALR* mutation. Additionally, occurrence of new bone marrow fibrosis was confirmed in seven *JAK2*-unmutated patients at the end of the study. In summary, in young patient population, we recommend limiting the use of anagrelide to *JAK2*-mutated subgroup, reducing exposure time and underline the importance of periodic monitoring for the presence of bone marrow fibrosis.

1. Introduction

Essential thrombocythemia (ET) is a chronic disease in which clonal megakaryocytic proliferation leads to elevated platelet (PLT) count and exposes affected patients to increased risk of both thromboembolic and bleeding complications, with an additional potential of disease transformation to secondary myelofibrosis or acute leukemia [1,2]. In the majority of cases the disease's clonal nature can be confirmed by the detection of specific mutation in one of the three driver genes - *JAK2, CALR,* or *MPL*. Patients diagnosed with ET present variable phenotype and several studies have proven that the clinical features and biology of ET are dependent on the type of mutation [3–7]. Moreover, based on the reports of polycythemic transformation, a phenomenon occurring only in patients carrying the *JAK2V617F* mutation, some authors even suggest that *JAK2*-mutated ET and polycythemia vera (PV) are two phenotypes of the same disease [3,4]. However, to date it remains in question whether the disease's molecular background have an actual impact on response to treatment.

The most recent treatment recommendations for ET suggest a risk-adapted approach, allowing observation only in low-risk and cytoreduction in high-risk patients [8]. Options for first-line cytoreductive agents include hydroxyurea (HU) or pegylated interferon alpha-2a, which use is emphasized especially in young patient population [9]. In patients experiencing either intolerance or resistance to first-line treatment, anagrelide (ANG), an oral imidazole-quinazoline derivative agent, believed to exert selective anti-megakaryocytic effect, is recommended as second-line approach. However proven non-inferior to HU in preventing thromboembolic complications [10], it has been observed that prolonged exposure to ANG can lead to clinically relevant anemia [11]. Direct inhibitory effect on erythroid progenitors has been suggested, but not confirmed in *in vitro* studies [12]. On the other hand, observations of clinical usage of other quinazoline derivatives revealed that a drop in Hb

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concentration may be caused by diminished sensitivity of erythroid precursors to erythropoietin (EPO) [13], however the exact mechanism underlying this complication remains unknown.

The correlation between the type of driver mutation and efficacy of ANG therapy is a subject of few publications [4,14,15]. Published reports are based on heterogeneous cohorts of patients with relatively short-term exposure to ANG. In this study we evaluate the time-related efficacy of ANG therapy in young ET patients who were exposed to ANG treatment for at least 10 years. Additionally, we aim to further investigate the possible mechanism of anemia caused by ANG by comparing the relationship between hemoglobin (Hb) concentration and serum EPO in patients with different driver mutations.

2. Patients and methods

2.1. Patients

All patients with the diagnosis of ET who were under the care of the Department of Hematology and Transplantology, Medical University of Gdańsk (Gdańsk, Poland) and who received ANG treatment, have been taken into consideration for the inclusion into the study group (n = 69). Since this study is investigating long-term effects of ANG exposure, patients in whom the treatment was not able to be continued for at least 10 years have been excluded from the study group. Treatment discontinuation rate was 19% in the first 5 years, and 14% in the following 5 years of treatment. Finally, 48 patients who were started with ANG in years 2000–2007 were qualified for the evaluation. Flow-chart representing the process of selection of the study group is presented in Fig. 1. The general characteristics of the study group is presented in Table 1.

The diagnosis of ET was based on Polycythemia Vera Study Group criteria [16], and then verified with 2008 and 2016 WHO criteria [17,18]. All patients included into the study group were below the age of 60. Patients have been qualified for cytoreductive treatment considering guidelines, local practice, and physician choice, based on medical history of thromboembolic (deep vein thrombosis n = 3, transient ischemic attack n = 1, pulmonary embolism n = 1, Budd-Chiari syndrome n = 2) or bleeding (n = 2) complications and/or PLT count over 1000 G/L (*CALR n = 25, JAK2 n = 14*). In 41 patients ANG was given as a second-line treatment after discontinuation of HU. Previous exposure to HU ranged from 2 to 18 months. Reasons for HU discontinuation included premature menopause symptoms (n = 19), resistance (n = 15), toxicity (n = 5), skin changes (n = 1) and fever (n = 1). In the remaining 7 patients ANG was used as a first-line treatment because of planned pregnancy in the future. The starting dose of ANG was 0.5 mg BID, followed by the dose titration up to maximum daily dose of 2.5 mg, based on the PLT response.

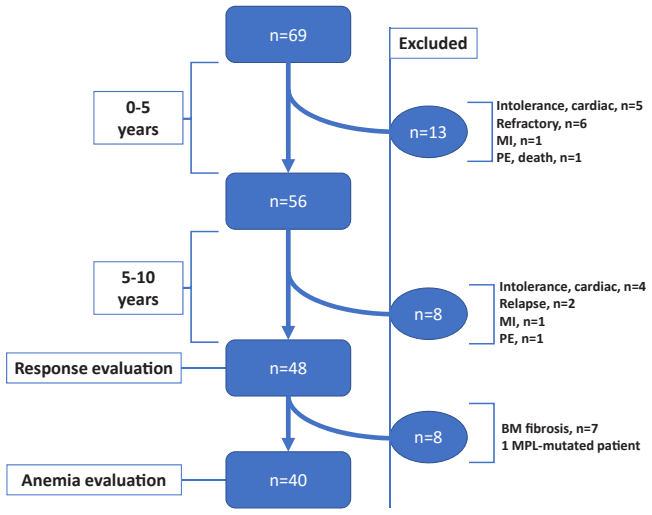

Fig. 1. Flow chart representing the process of selection of the study group. MI – myocardial infaction; PE – pulmonary embolism; BM – bone marrow.

Table 1
General characteristics of the study group (TN – triple negative).

	JAK2	*CALR*	*MPL*	TN
N	17	23	2	6
Sex (F:M)	15:2	18:5	2:0	6:0
Age at diagnosis, mean (range)	32,4 (16 – 46)	35,4 (19–48)	37	32,8 (22–46)
Time from diagnosis to evaluation, years (range)	13,6 (10–20)	14,2 (10–17)	13	15 (11–21)
ANG treatment, years (range)	12,5 (10–17)	12,8 (10–17)	12	13,6 (11–17)
ANG 1st line treatment	4	2	0	1
Parameters at diagnosis median (range)				
Hb, g/dl	14,1 (13–15,8)	13,1 (12,1–14,2)	13,4 42	13,2 (12,2–14,2)
Hct, %	39,5 (39–46)	39 (37–45)	1482	38 (37–46)
PLT, G/l	1349 (771–1970)	1292,5 (706–3290)	8,4 24,8	1171 (961–1559)
WBC, G/l	9,21 (5,67–13,48)	9,67 (4–13,3) 17,4		9,84 (8,49–11,1)
EPO, mU/mL	6,3 (2,1–17,6)	(5,8–60,2)		25,1 (6,2–66,3)

2.2. Methods

Patients included in the study group have been evaluated regarding mutational status, bone marrow fibrosis, treatment response and serum EPO.

At diagnosis, all patients underwent screening for the presence of *JAK2V617F* mutation [19]. In 2014, all patients with *JAK2*-negative ET were examined for *CALR* and *MPL* mutations [20,21].

Complementary histopathological bone marrow examination was performed to assess the fibrosis grade twice during the study – at a 5-year and 10-year interval.

Treatment response was evaluated in the 5th and 10th year of treatment (+/- 6 months). Response was established based on the 2009 European Leukemia Net (ELN) criteria and verified with revised criteria published in 2013 [22,23].

Changes in Hb concentration throughout the study and serum EPO after 10 years of ANG treatment have been evaluated in 40 patients (*JAK2* n = 17, *CALR* n = 18, TN n = 5). Patients with confirmed progressive fibrosis (n = 7) and the one remaining *MPL*-mutated patient have been excluded from anemia evaluation to avoid bias (Fig. 1). Alternative causes of anemia, including bleeding, hemolysis, iron, B12 or folate deficiency has been excluded in patients developing progressive anemia, as per local practice.

2.3. Statistical analysis

The analysis was performed using STATISTICA 12.0 software. Significance of differences in continuous variables between 2 groups was analyzed by either Student's *t*-test or Mann–Whitney *U*-test. For comparison of 3 groups 1-way ANOVA followed by Tukey multiple comparisons test or Kruskal–Wallis's test were used. Chi-square or Fisher exact test were used to analyze categorical variables. Correlation analysis was performed using Spearman's test. Two-sided *P*-values < 0.05 were considered significant.

3. Results

The *JAK2V617F* mutation was detected in 17 patients (35%), *CALR* exon 9 mutation in 23 (48%), and *MPL* mutation in 2 (4%). In 6 patients

(13%), no molecular marker was found (triple negative, TN).

In evaluated patients, at the 5-year interval, proportions of complete remission (CR) according to the 2009 ELN criteria achieved in *CALR*, *JAK2*, *MPL*-mutated and TN groups were similar (Fig. 2). The therapy was well tolerated, treatment-related adverse events as well as disease's specific complications (bleeding, thrombosis) were not observed. At that time, bone marrow biopsy revealed no features of fibrosis and megakaryocytic dysplasia. However, in all evaluated patients the proportion of erythroid precursors in bone marrow was slightly decreased.

At the 10-year interval, no patients from the study group were labeled as no remission (NR) according to the 2009 ELN criteria (Fig. 3A) and rates of CR were comparable between *JAK2*- and *CALR*-mutated patients ($p = 0.1$). However, when revised criteria published in 2013 were used, 3 patients were labeled as NR (Fig. 3B), which resulted in the decrease in CR from 67% (ELN 2009) to 58% (ELN 2013) in *CALR*-mutated group and significant difference compared to *JAK2*-mutated group ($p = 0.03$). At this study point, the presence of reticulin fibrosis in bone marrow biopsy was found in 7 patients (*CALR* $n = 5$, *MPL* $n = 1$, TN $n = 1$) and was the reason for lower proportions of CR in *CALR*-mutated subgroup.

In the remaining 40 evaluated patients, mean Hb concentration, both at the time of diagnosis and start of ANG therapy, in the *CALR*-mutated subgroup was significantly lower than in the *JAK2*-mutated subgroup ($p = 0.0013$ and $p = 0.0001$, respectively). A similar difference was found after 5 and 10 years of treatment ($p = 0.007$ and $p < 0.0001$, respectively). At a 5-year interval, a significant decrease in Hb concentration was observed in all patients ($p = 0.0004$ for *JAK2*, $p = 0.0001$ for *CALR*, $p = 0.04$ for TN) and there was no correlation between the degree of Hb concentration decrease and type of mutation. However, at a 10-year interval, only patients harboring *CALR* mutation were characterized by a further, progressive, Hb concentration decrease when compared to other mutational groups ($p > 0.05$ for *JAK2* and TN, $p = 0.0001$ for *CALR* group) (Fig. 4).

The dynamics of changes in Hb concentration during the study was assessed by calculating the difference between the values obtained at study entry, at 5-year and 10-year intervals. No significant differences in the rate of Hb concentration change were found in the first 5 years of treatment between patients with different mutational profiles. However, at a 10-year interval, patients with *CALR*-mutated ET had a significant Hb concentration decrease ($p < 0.01$) in comparison to patients with *JAK2*-mutated and TN ET (Supplementary Fig. 1).

The analysis of serum EPO after 10 years of treatment indicated a significantly lower concentration in patients with *JAK2*-mutated ET in comparison to patients with *CALR*-mutated and TN ET (Fig. 5). No correlation between the values of EPO and Hb concentrations was found for both the whole analyzed group, *JAK2*-mutated and TN ET subgroups. However, in the patients carrying the *CALR* mutation, a strong negative correlation was noted ($p < 0.01$) (Supplementary Fig. 2).

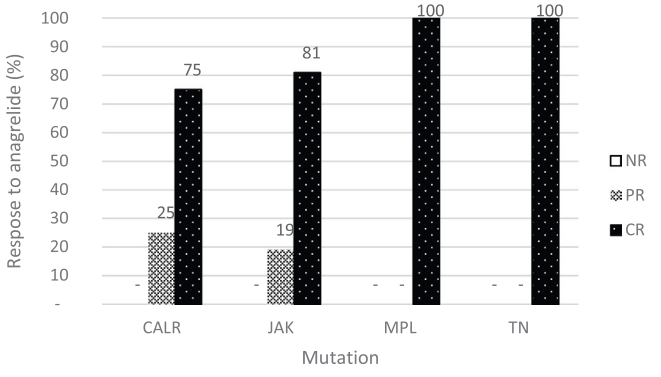

Fig. 2. Response at 5 years of ANG treatment according to ELN 2009.

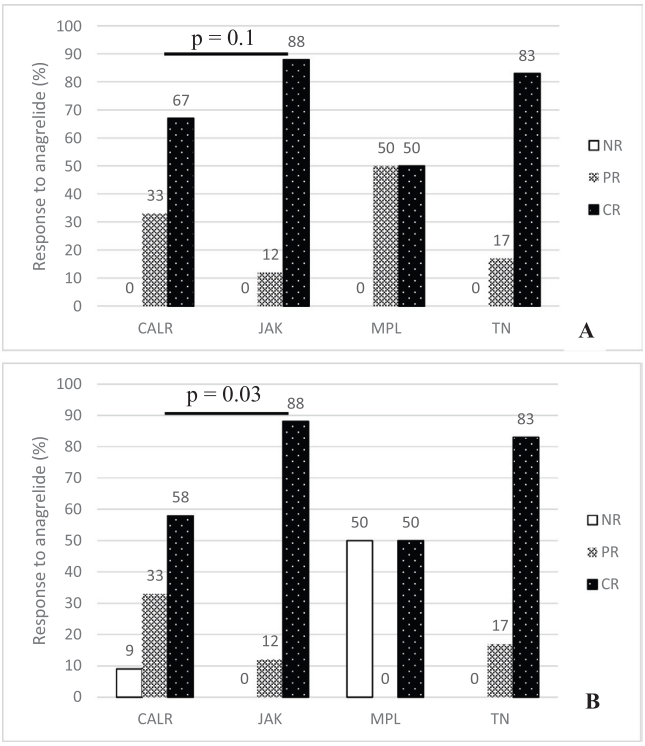

Fig. 3. Response after 10 years of ANG treatment. A – ELN 2009. B – ELN 2013.

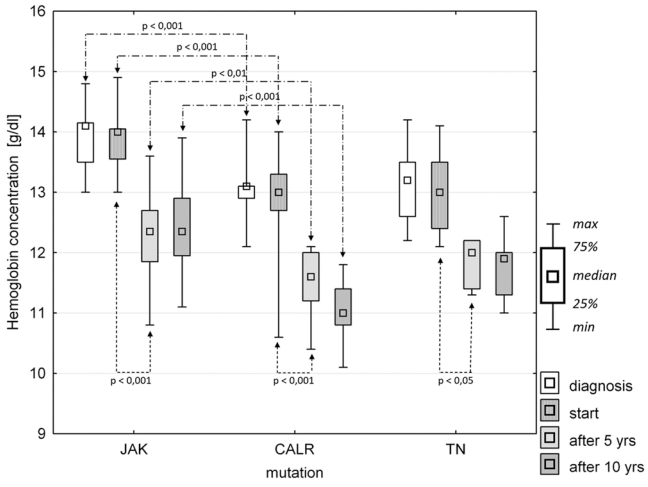

Fig. 4. Mean Hb concentration (g/dl) at study timepoints based on mutational status.

4. Discussion

ANG has proven effectiveness in the treatment of high-risk ET patients [10]. According to ELN, it is currently recommended as a second-line option in patients unresponsive or intolerant to HU or pegylated interferon alpha-2a [9]. Moreover, because of suspected lower leukemogenic risk compared to HU, its use is encouraged in younger patient population requiring cytoreduction [24]. In our study group, at the start of ANG treatment, the median age of patients was below 50 years, with only 1 patient exceeding the age of 60 throughout the study. The distribution of driver mutations in our cohort does not represent their distribution seen in the general ET population, where *JAK2* mutation is expected to be detectable in over 60% of patients [3]. The majority of patients included into the study (85%) received ANG as

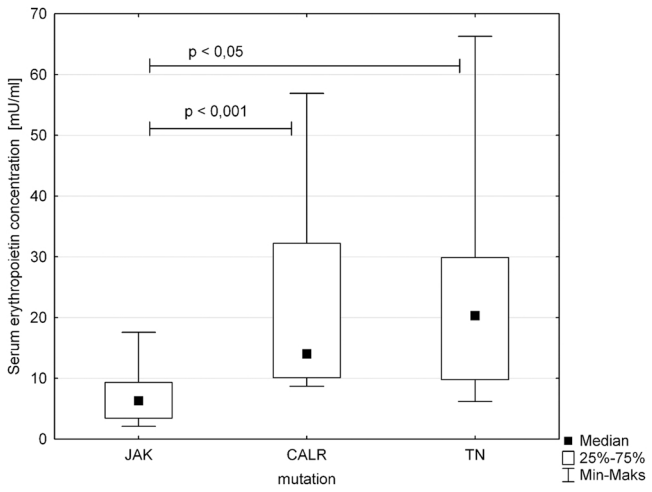

Fig. 5. Median serum EPO at 10 years of treatment.

a second-line therapy and as much as 39 patients had PLT counts > 1000 G/l at study entry, hence could be considered refractory to previous treatment. According to Campbell et al. patients diagnosed with *JAK2*-mutated ET are characterized by better response to cytoreductive treatment, especially HU [4]. Therefore, the need for the introduction of second-line treatment is expected to be more apparent in patients harboring *CALR* mutation, who comprised almost 50% of our study group.

We presented the evaluation of the response to ANG treatment at different points of the study. At a 5-year interval, the evaluation did not show any differences in rates of achieving CR, regardless of type of driver mutation. This is in line with research conducted by Osorio et al. and Iurlo et al., however based on small study groups and heterogenous treatment time [15,25]. The second evaluation made at a 10-year interval shows that the highest percentage of CR was seen in patients with *JAK2* mutation and was independent of the criteria that were used. Patients harboring *CALR* or *MPL* mutation or TN presented with lower response rates compared to *JAK2* subgroup, with proportion of CR diminishing throughout the study as well as with the use of more strict criteria.

In the presented data, the assessment of CR with the use of ELN 2009 [22] and ELN 2013 [23] criteria differs by 10% (Fig. 2). The lower proportion of CR is a result of the inclusion of the bone marrow assessment in the latter, particularly the requirement for the absence of fibrosis graded as MF-1 or higher. In our cohort, after 10 years of treatment with ANG, reticulin fibers were detected in 7 patients, none of them harboring *JAK2* mutation. Noteworthy, none of the patients included into the study group had histopathological features suggesting prefibrotic MF. It was previously reported that the onset of marrow fibrosis in ET patients may be accelerated by ANG treatment, particularly in *CALR*-mutated patients [15,26]. On the other hand, rates of fibrotic transformation in patients treated with HU are significantly lower as compared to those treated with ANG [27,28]. Moreover, treatment with PEG-IFN-a2a was shown to even resolve bone marrow fibrosis in large subset of patients [29]. Our observations, despite small study sample, confirm previous results, that patients without *JAK2* mutation are more susceptible to profibrotic effects of ANG. Hence, prolonged treatment with ANG in *JAK2*-unmutated patients should be planned with caution and periodic assessment for bone marrow fibrosis should be considered.

In our study group, patients with *CALR*-mutated ET had lower median Hb concentration as compared to *JAK2*-mutated ET both at diagnosis and study entry ($p < 0.001$). This observation confirms previously published findings from study conducted on larger patient cohort, where Rumi et al. analyzing differences driven by molecular profile in ET population revealed similar differences in baseline Hb in *JAK2*- vs *CALR*-mutated ET ($p < 0.001$) [30]. The effect is expected to be a result of different molecular mechanisms driving the disease in patients harboring these mutations. Moreover, the existence of *JAK2*-mutated ET was questioned, suggesting it being an early stage of PV (masked PV) [31]. No patient included in our study fulfilled the criteria of masked PV.

All patients exposed to ANG treatment in this study had a significant decrease in Hb concentration over the study period. Progressive anemia in ANG-treated patients has been described previously in large population studies [32]. However, the mechanism behind this complication remains uncertain. Gisslinger et al. in their work reported anemia as one of adverse events in patients with ET treated with ANG as well as with HU [10]. In another report analyzing long-term effects of ANG in young ET patients, authors noticed that anemia was the only new emerging side effect, with 24% patients experiencing as much as 3 g/dl decrease in hemoglobin concentration. Authors state that mechanism of anemia is unknown but they speculate it may be the result of induced erythropoietin resistance, since in patients who discontinued ANG or received exogenous EPO, resolution of anemia was observed [11]. Another study, evaluating over 5-year ANG treatment period in young ET patients, revealed development of anemia in only 4 patients (15%), two with moderate and two with severe (<8 g/dl) anemia. At diagnosis, bone marrow biopsies of those patients were not showing features suggestive for prefibrotic MF. However, bone marrow biopsies taken at the time when anemia was detected, showed progressive fibrosis in 2 patients with moderate anemia and erythroblastic hypoplasia without fibrosis in the remaining 2 patients with severe anemia. Additionally, former 2 patients had elevated EPO levels (120 mU/mL and 66 mU/mL) [33]. Unfortunately, in all aforementioned papers, the distribution of driver mutations among the study subgroups is unknown. Osorio et al. presented mutation-oriented retrospective analysis of 67 patients treated with ANG. Authors reported anemia as the major adverse event which was present in 30,3% of study group at 5-years follow-up. Interestingly, anemia was seen more frequently in *CALR*-mutated group ($p < 0.05$). However, it must be taken into consideration that 21% of *CALR*-mutated patients developed myelofibrotic transformation which might have contributed to progressive anemia [15].

Findings from our study are consistent with previous reports, however these reports were not focused particularly on mechanisms of anemia, did not always clearly state the distribution of mutational status among the study group and did not exclude patients with features suggesting prefibrotic phase of MF or progression of bone marrow fibrosis. We were able to confirm progressive anemia in all patients treated with ANG, without features of progressive bone marrow fibrosis and regardless of driver mutation in the first 5 years of the study. However, only in patients harboring *CALR* mutation this trend was sustained for the whole 10-year observation period.

Following the idea of reduced sensitivity of erythroid progenitors to external stimulation [13], we decided to additionally evaluate serum EPO concentration in each patient at the end of the study. In patients with *JAK2*-mutated ET, there is a possibility that erythroid progenitors are also burdened with the mutation, hence being able to retain the EPO-independent growth. In our cohort, patients with *JAK2* mutation presented with significantly lower serum EPO compared to other groups, had higher Hb concentrations at every evaluation and resisted further decline in Hb at 10-year interval. On the other hand, patients harboring *CALR* mutation presented with lower Hb, had sustained decline in Hb concentrations throughout the study and most interestingly, showed negative correlation between serum EPO and Hb concentration. Those findings suggest that patients treated with ANG develop diminished susceptibility to EPO over time, whereas patients harboring *JAK2* mutation show partial resistance to this complication, presenting with features similar to those seen in PV.

There is an ongoing dilemma concerning the choice of cytoreductive agent that should be used in the population of young MPN patients. Moreover, it is disputed whether the benefits gained from introducing

cytoreduction outweighs the potential long-term consequences. In our work we presented an analysis of 48 young patients diagnosed with ET that have been exposed to cytoreductive treatment for a great portion of their life. The reason for starting cytoreduction included history of thromboembolic complications, but in the majority of patients it was dictated by the presence of extreme thrombocytosis (ExT), defined as PLT count> 1000 G/l. Similar findings were reported by Gangat et al. in their work on young ET patients, where cytoreduction was willingly introduced, especially in the presence of ExT [34]. According to the currently proposed treatment guidelines, ExT as a sole abnormality is not placing the patient in the high-risk group and should not be regarded as an indication for treatment [8,9]. However, aforementioned reports and our own experience show that in a real-life setting, there is a doubt whether it is safe to keep young patients with a diagnosis of clonal disease only in observation. This doubt might be strengthened by the findings in a recently presented work by Abu-Zeinah et al. [35] who, contrary to common belief, revealed an excess mortality in young MPN patients as compared to the healthy population of the same age. If, despite the recommendations, cytoreductive treatment is willingly introduced in young patients, it is of the most importance to focus on careful selection of treatment agents.

In our work we presented not only the efficacy of ANG treatment, but also possible long-term consequences, including bone marrow fibrosis and progressive anemia, unrelated to fibrosis, in *CALR*- but not in *JAK2*-mutated ET patients. The major disadvantage of this study is relatively small patient cohort, hence future studies, ideally on larger patient cohorts in multicenter setting, are needed. Nevertheless, we encourage to take our findings into consideration when choosing therapeutic options in this population.

Ethics approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Funding

No funding was received for conducting this study.

Statements and declarations

None.

Informed consent

Informed consent was obtained from all patients for being included in the study.

Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.leukres.2022.106962.

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The molecular profile in patients with polycythemia vera and essential thrombocythemia is dynamic and correlates with disease's phenotype

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Introduction: Polycythemia vera (PV) and essential thrombocythemia (ET) are diseases driven by canonical mutations in JAK2, CALR, or MPL gene. Previous studies revealed that in addition to driver mutations, patients with PV and ET can harbor other mutations in various genes, with no established impact on disease phenotype. We hypothesized that the molecular profile of patients with PV and ET is dynamic throughout the disease.

Methods: In this study, we performed a 37-gene targeted next-generation sequencing panel on the DNA samples collected from 49 study participants in two-time points, separated by 78-141 months. We identified 78 variants across 37 analyzed genes in the study population.

Results: By analyzing the change in variant allele frequencies and revealing the acquisition of new mutations during the disease, we confirmed the dynamic nature of the molecular profile of patients with PV and ET. We found connections between specific variants with the development of secondary myelofibrosis, thrombotic events, and response to treatment. We confronted our results with existing conventional and mutation-enhanced prognostic systems, showing the limited utility of available prognostic tools.

Discussion: The results of this study underline the significance of repeated molecular testing in patients with PV and ET and indicate the need for further research within this field to better understand the disease and improve available prognostic tools.

KEYWORDS

polycythemia vera, essential thrombocythemia, molecular profile, thrombosis, secondary myelofibrosis, next-generation sequencing

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1 Introduction

Myeloproliferative neoplasms (MPN) are driven by specific mutations in one of the three genes – *JAK2, MPL* or *CALR* (1). These mutations cause uncontrolled activation of the JAK-STAT signaling pathway leading to the release of secondary mediators of pro-survival and proliferative nature (2).

The rapid development of sequencing techniques in recent years allowed for better characterization of molecular profiles in patients with hematological malignancies and significantly contributed to an understanding of disease biology. It is accepted that besides driver mutations, the molecular profile of MPN comprises variants in a variety of genes (3, 4). The presence and allelic burden of those mutations are suspected to impact the disease's clinical course. However, in MPNs, the chronic nature of the disease hinders the research aimed at understanding the significance of molecular changes, contrary to entities of acute nature. Mutations in only a fraction of genes have been recently proposed as potentially influencing disease risk and included in mutation-enhanced international prognostic systems (MIPSS) for polycythemia vera (PV) and essential thrombocythemia (ET) (5).

In this study, we aim to assess the dynamics of the molecular profile in PV and ET by investigating the occurrence and allelic burden of both driver and passenger mutations at two-time points. We hypothesize that the molecular profile of patients with PV and ET evolves with time, accumulating mutations in expanding malignant clones. The chronic nature of those diseases implies the need to assess the risk multiple times. We believe studies on the topic may help predict patient outcomes in advance, giving the time to react and treat accordingly.

2 Patients and methods

Study participants have been selected from the population of patients of the Outpatient Ward, Department of Hematology and Transplantology, Medical University of Gdansk, Poland. Initially, all alive patients diagnosed with PV or ET were identified. Next, individuals for whom the diagnosis was made at least five years prior to the study were selected. Among those patients, we identified individuals from whom DNA samples of sufficient quality and quantity were available from the time of diagnosis. Finally, 49 patients consented to participate in the study.

Diagnoses were verified with MPN 2016 WHO criteria (6). Two patients with apparent features of myeloproliferative neoplasms but undetected driver mutation were labeled triple-negative (TN). Each patient's documentation was analyzed to collect data regarding treatment, thrombotic complications, and disease progression. The general characteristics of the study group are presented in Table 1.

A peripheral blood sample was collected from each participant for molecular testing and comparison with corresponding archival samples. The median time between historical sample collection and a present sample was 104 months (range 78-141 months)

Genomic DNA was extracted from peripheral blood samples using QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions. DNA was quantified by spectrophotometric method and stored at –20°C for further analysis.

Next-generation sequencing was performed using the Archer VariantPlex Core Myeloid kit (ArcherDX), Mid Output Kit (300-cycles), and MiniSeq (Illumina). It allowed performing a comprehensive analysis of 37 genes: *ABL1, ANKRD26, ASXL1, BCOR, BRAF, CALR, CBL, CABPA, CSF3R, DDX41, DNMT3A,*

TABLE 1 General characteristics of the study group.

	1st sample	2nd sample
PV		
N	13	
Sex, F/M	7/6	
Sample collection, months (range)	Diagnosis	104 months (83 -134)
Age, years, median (range)	57 (24 - 68)	66 (34 - 77)
Hb, g/dl, median (range)	17,9 (14,5 - 22)	14,9 (12 – 16,7)
PLT, G/l, median (range)	507 (315 - 906)	319 (181 – 744)
WBC, G/l, median (range)	9,49 (6,26 - 21,12)	5,92 (4,04 – 26,71)
LDH, U/L, median (range)	186 (147 – 442)	221 (146 – 796)
Cytoreduction/Observation	11/2	13/0
Treatment change (%)	n/a	3 (23)
Thrombosis (%)	3 (23)	2 (15)
Risk low/high	6/7	n/a
MIPSS low/int/high	11/2/0	5/8/0
Progression to MF (%)	n/a	2 (15)
Progression to AML (%)	n/a	0
ET		
N	36	
Sex, F/M	23/13	
Sample collection, months (range)	Diagnosis	104 months (78-141)
Age, years, median (range)	54 (28 – 67)	63 (35 – 74)
Hb, g/dl, median (range)	13,9 (10 – 16,4)	13,5 (8,8 – 17)
PLT, G/l, median (range)	760 (516 – 1750)	425 (22 – 824)
WBC, G/l, median (range)	7,9 (4,9 – 25,9)	6,5 (3,4 – 19,3)
LDH, U/L, median (range)	209 (129 – 473)	222 (128 – 1056)
Cytoreduction/Observation	28/8	32/4
Treatment change (%)	n/a	17 (47)
Thrombosis (%)	2 (5)	4 (11)
Risk low/high	26/10	n/a
MIPSS low/int/high	27/5/4	13/12/11
Progression to MF (%)	n/a	7 (19)
Progression to AML (%)	n/a	0

n/a, not applicable.

ETNK1, ETV6, EZH2, FLT3, GATA1, GATA2, IDH1, IDH2, JAK2, KIT, KRAS, MPL, NPM1, NRAS, PHF6, PTPN11, RUNX1, SETBP1, SF3B1, SRSF2, STAG2, TET2, TP53, U2AF1, WT1, ZRSR2. The results were analyzed using Archer Analysis v.6.0.3.2 software (ArcherDX), and 2,7% allele frequency (VAF) as a cutoff was applied.

Collected data was used to stratify patients into the risk groups during sample collection. Conventional risk stratification was made according to the revised IPSET-T system in ET and based on age and thrombotic complications in PV (7, 8). Acquired sequencing data was used to attribute patients into the low- intermediate- or high-risk groups according to MIPSS-ET or MIPSS-PV (5). Since cytogenetic data were incomplete in the study population, all PV patients were considered to have normal karyotypes.

An additional exploratory risk stratification, including 5- and 10-year event-free survival (EFS), risk of secondary myelofibrosis (sMF) or acute myeloid leukemia (AML) were assigned based on prognostication model developed by Grinfeld et al. (4). This stratification was made to assess the utility of the proposed MPN Personalized Risk calculator (available online at https://www.sanger.ac.uk/science/tools/progmod/progmod/). For this evaluation, all patients were considered unknown karyotypes.

Statistical analysis was performed using the computer program STATISTICA v.13.3. Data was statistically described in terms of mean or median and range. Comparison between the two groups was made using Mann–Whitney U test for continuous variables and the Chi-square test for categorical variables. P values were considered significant if less than 0.05.

3 Results

3.1 General findings

A total of 98 samples were analyzed, including 49 archival samples and 49 follow-up samples. In summary, 78 variants were detected among the analyzed 37 genes (Supplementary Table 1). The most frequently detected variants in driver genes were *JAK2* p.Val617Phe (32 pts), *CALR type 1* (8 pts), *CALR type 2* (5 pts) and *MPL* p.Trp515Leu (2 pts). The gene plot showing detected mutations in analyzed genes across 1st and 2nd samples of each patient is presented in Figure 1. New variants detected on the 2nd sample are highlighted in red. The dynamics of VAF of driver mutations is shown in Figure 2A.

In contrast with the paradigm that driver mutations in MPNs are mutually exclusive, our analysis revealed two coexisting canonical mutations in one patient (#16) – *MPL* p.Trp515Leu variant (VAF 14,02%) and *JAK2* p.Val617Phe variant (VAF 5,17%) at diagnosis. In the follow-up sample, only the *MPL* p.Trp515Leu variant was detectable (VAF 9,3%).

Non-canonical variants in driver genes were detected in four patients. One patient (#2) had non-canonical driver mutation in *MPL* gene – p.Ser505Asn c.1514G>A – present at diagnosis and follow-up, with VAF of 31,92% and 27,31%, respectively, and coexisting with *JAK2* p.Val617Phe, detectable only in a second sample at relatively low VAF (6,47%). In the remaining three patients, non-canonical variants in driver genes also coexisted with canonical mutations and included *JAK2* p.Lys607Asn c.1821G>T detected along with *JAK2* p.Val617Phe (#14), *MPL* c.1565 + 5C>T detected along with *JAK2* p.Val617Phe (#47) and *MPL* p.Ser493Phe c.1478C>T was observed along with *MPL* p.Trp515Leu (#22). No non-canonical mutations in *CALR* were detected.

In three female ET patients receiving hydroxyurea monotherapy (#9, 16, and 47), *JAK2* p.Val617Phe mutation was detectable at diagnosis but not at follow-up. Here, the variant was initially detected at relatively low VAF (5,17-8,9%) and probably reduced below the detection threshold at follow-up (<2,7%). Several other mutations were undetectable in follow-up samples (Figure 1; Supplementary Table 1). Those variants were detected at low VAFs at diagnosis and did not have confirmed reports of pathogenicity.

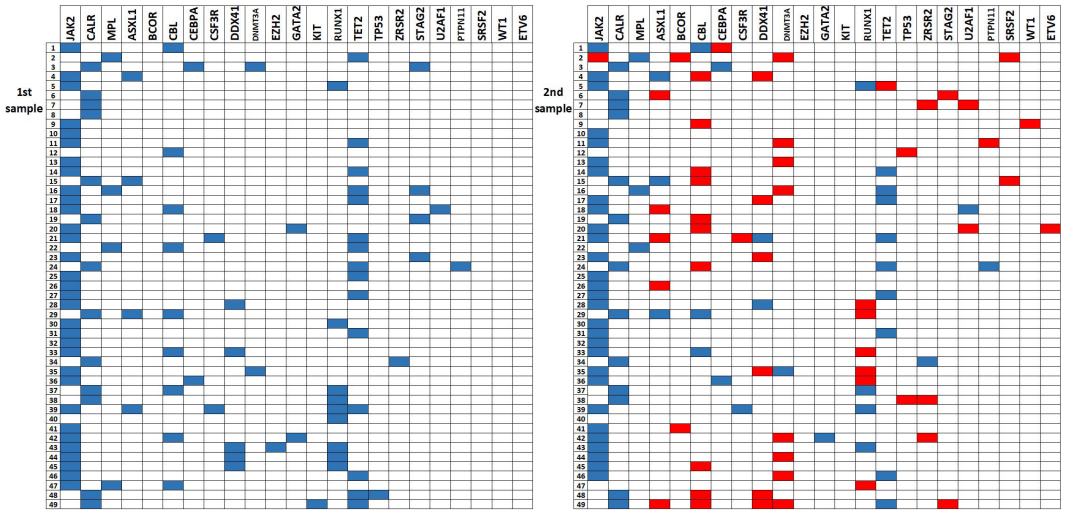

FIGURE 1
The gene plot showing detected mutations in analyzed genes across 1st and 2nd samples in each patient. New mutations in 2nd sample are highlighted in red.

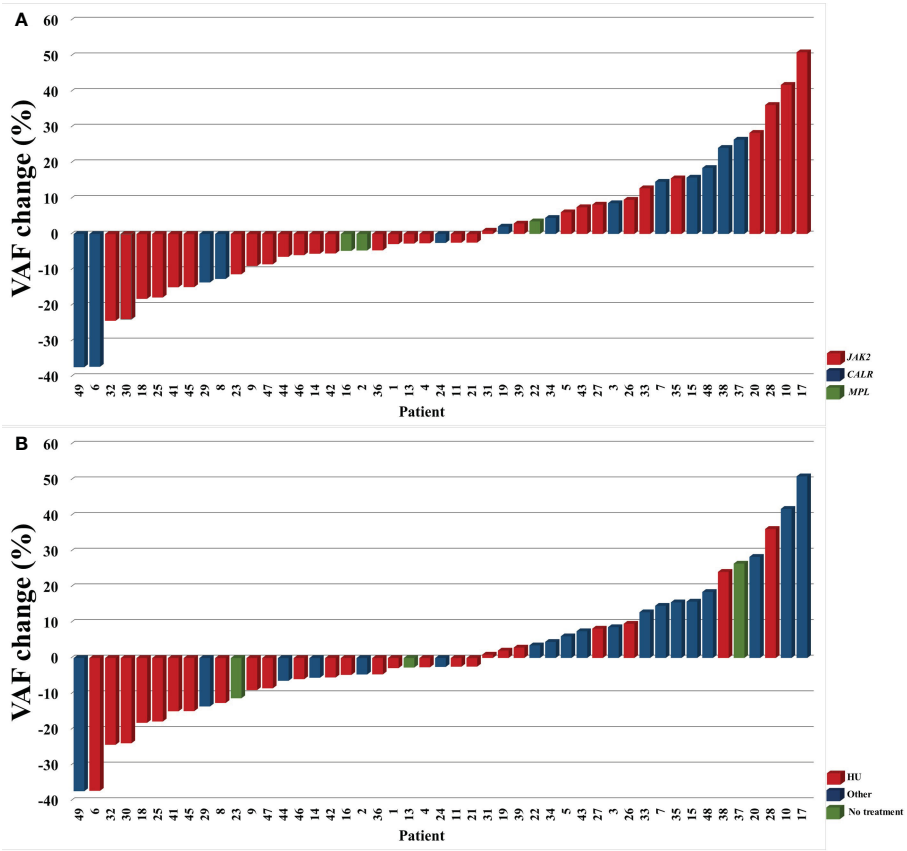

FIGURE 2
The dynamics of VAF of driver mutations in analyzed subgroups by the type of driver mutation **(A)** and treatment **(B)**. TN patients (#12 and 40) are not included.

Among TN patients (#12 and 40) we detected variants with low VAFs, including CBL p.Asp460del c.1380_1382del variant (VAF 2,75%), RUNX1 p.Arg423Gly c.1267C>G (VAF 3,72%) variant and pathogenic TP53 p.Arg248Leu c.743G>T (VAF 6,96%) variant.

Among non-driver genes, the most frequently detected variants were *CBL* p.Asp460del c.1380_1382del (19 patients), *RUNX1*p.Glu422Ala c.1265A>C (11 patients), *DDX41* p.Val408Asp c.1223T>A (9 patients) and *STAG2* p.Glu342Ter c.1024G>T (5 patients). Those variants have not established pathogenicity.

Since in our study population, the time to the 2nd sample acquisition ranges from 78 to 141 months (median 104 months), we wanted to investigate whether there is a difference in the number of variants detected on 2nd sample in patients with longer observation time (above median) compared to patients with shorter observation time (below median). The mean number of variants was higher in patients with longer observation times, with a borderline significance of p=0.049 (Figure 3A). We also performed the analysis to verify whether some variants detected in the second sample correlate with disease progression. A number of variants detected in both diagnosis and follow-up samples were similar in patients requiring treatment change, experiencing thrombotic complications and developing fibrotic progression (p>0.05 for all comparisons).

The two most frequently mutated genes were *TET2* (18 variants) and *DNMT3A* (ten variants). We observed a high prevalence of *TET2* mutations, both in diagnosis (n=15), with median VAFs of 46,99% (range 3,49 – 54,94%), and follow-up samples (n=10), with median VAFs of 49,02% (20,06 – 50,09%), suggesting its germline origin. Patients harboring *TET2* mutation in 1st sample were older (median of 59 years, compared to 54 for the rest of the group). However, this difference was not statistically significant (p=0.12). On the other hand, *DNMT3A* mutations were rarely detected at diagnosis (n=2) with median VAFs 9,22% (8,03 – 10,41%) but frequently at follow-up samples (n=9) with median VAFs of 4,7% (3,53 – 25,01%), corresponding with somatic origin. Patients that acquired *DNMT3A* mutations had median time between sample collection of 102 months, below the median for the whole group. Moreover, patients with *DNMT3A* mutation emerging in the second sample (n=8), had median age of 64 years, compared to 65 years for the rest of the group. Those findings indicate that acquiring DNMT3A mutations is not connected with the duration of treatment or age. Next, we confronted whether specific patterns of *DNMT3A/TET2* mutation dynamics correlated with the clinical picture. *DNMT3A/TET2* mutations were rarely observed in patients developing sMF (at diagnosis none with *DNMT3A* and four with *TET2*, at follow-up one with *DNMT3A* and two with *TET2*). Four out of eight emerging *DNMT3A* mutations were detected in patients receiving hydroxyurea (HU) monotherapy. On the other hand, VAFs of

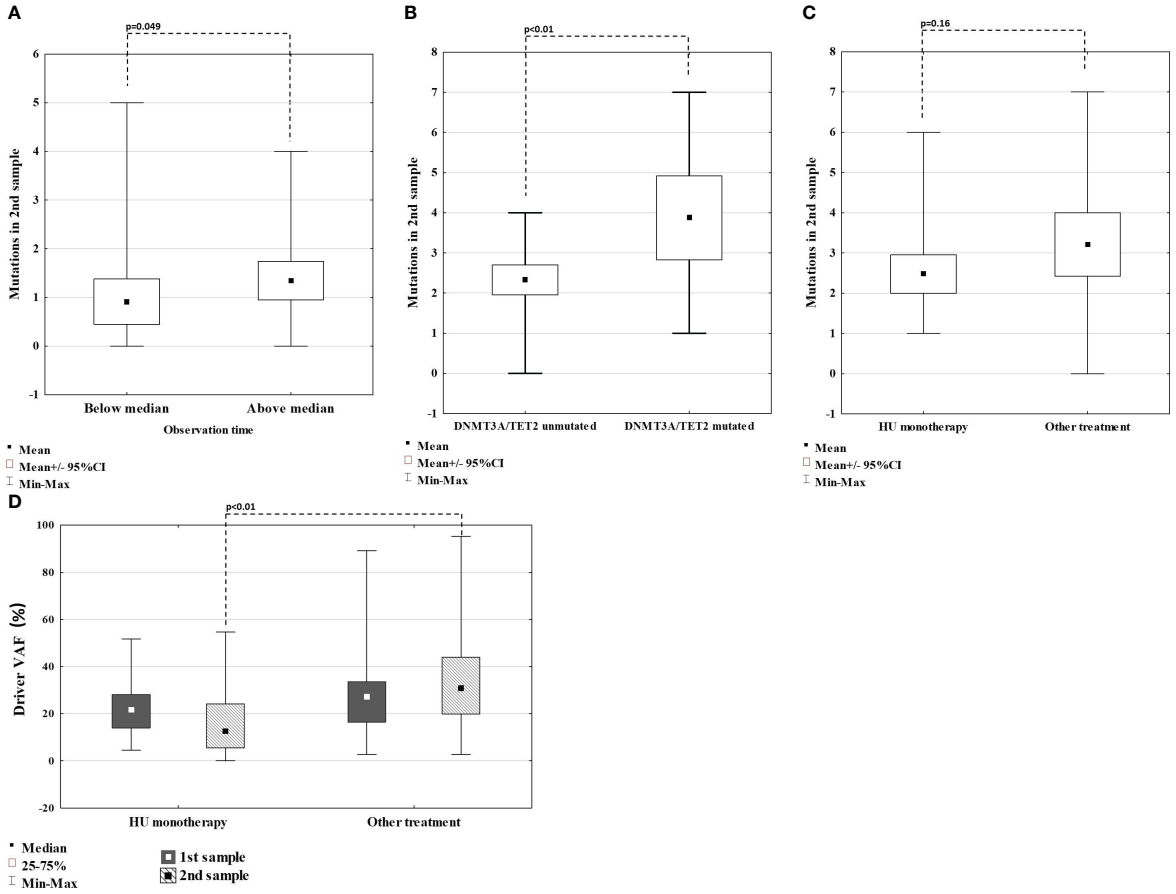

FIGURE 3
Comparison of number of mutations detected on 2nd sample in regard of time between sample collection **(A)**; in patients with and without *TET2* or *DNMT3A* mutation **(B)**; in patients treated with HU monotherapy **(C)**. Comparison of VAF of driver mutation in 1st and 2nd sample in patients treated with HU monotherapy vs others **(D)**.

TET2 mutations were reduced (-0,18% to -19,84%) in patients on HU monotherapy. There were no differences in the presence or VAF of *DNMT3A/TET2* mutations in patients requiring treatment change or experiencing thrombosis compared to others. However, patients harboring *TET2* or *DNMT3A* mutations in the first sample had significantly more variants overall detected in the second sample, compared with *TET2* or *DNMT3A*-unmutated patients (Figure 3B). Additionally, one patient (#35) harbored *DNMT3A* p.Glu733Ala variant with VAF increasing across measurements from 10% to 23%, significantly higher than of other *DNMT3A* variants detected and this variant was previously connected with progression to AML.

During the study period, we observed emerging *ASXL1* mutations in five patients. Three patients (#18, 21, 49) acquired the p.Gly646TrpfsTer12 variant which was previously described in myelofibrosis and AML. Patient #18 developed myelodysplasia, and #49 developed sMF. Other variants included p.Pro808LeufsTer10 c.2421del (#26), previously described in MDS patients with fibrosis, and p.Thr736GlnfsTer8 (#6), undescribed previously.

3.2 Risk groups

Using the revised IPSET-T stratification system in ET patients at diagnosis, 26 were stratified to the non-high-risk group (very low - 10 patients, low - 16 patients), whereas 10 patients were attributed to the high-risk group. Among the high-risk patients, two had thrombotic complications (both myocardial infarction), and eight were above or equal to 60 years of age.

Using the conventional risk stratification in PV patients at diagnosis, six were attributed to the low-risk group, whereas seven were attributed to the high-risk group. Among high-risk patients three had thrombotic complications (two ischemic strokes, one deep vein thrombosis) and four were above or equal to 60 years of age.

The second assessment was performed by calculating the risk according to MIPSS-ET and MIPSS-PV at each sample collection. We also checked in which cases the MIPSS score was increased by findings from genetic analysis. In most patients, the MIPSS score increase between two consecutive samples was mediated only by age (17 patients in ET, six patients in PV). In the ET group, results from

the genetic analysis on the 2nd sample placed the patient in the high-risk group in as many as five cases. On the contrary, no such observation was made in the PV group, even though one young patient acquired a known pathogenic mutation in the *TP53* gene (p.Arg248Leu c.743G>T).

The final risk stratification was made using MPN Personalized Risk Calculator. Patients with PRV had significantly higher calculated median EFS (p<0.01), 5- and 10-year OS (p<0.01 for both), and lower 5- and 10-year AML risk (p<0.01 for both), both at diagnosis and follow-up when compared to ET patients. On the other hand, 5- and 10-year MF risk calculated at diagnosis and follow-up was comparable between ET and PV patients (p=0.7; p=0.8 and p=0.8; p=0.6, respectively). Results are presented in Figures 4A–C.

3.3 Treatment

At diagnosis, 38 (78%) patients were started with cytoreduction with HU, one patient (young female) received interferon, while ten patients were without cytoreductive treatment. During the time between each sample collection, 20 patients required treatment change, while 25 patients remained on treatment with HU. The reasons for treatment change were refractoriness to previous therapy (nine patients), the toxicity of prior treatment (seven patients), progression to sMF (two patients) and reaching the conventional high-risk group (two patients). At follow-up, patients were treated with various cytoreductive agents, including hydroxyurea, busulfan, anagrelide, peg-IFN-a2a, and ruxolitinib. Four patients were not receiving cytoreduction at follow-up - one patient with sMF treated symptomatically with blood transfusions and three patients who were not started with cytoreduction from the diagnosis.

Compared to other patients, patients receiving HU monotherapy across the study observation time had similar rates of developing sMF and thrombotic complications, a similar number of new mutations, mutations overall, and *DNMT3A/TET2* mutations detected on 2nd sample (p>0.05 for all comparisons) (Figure 3C). Out of five patients who acquired variants in the *ASXL1* gene at follow-up, four (80%) were treated with HU monotherapy (#6, 18, 21, 26). However, patients treated with HU had lower median VAF of driver mutation assessed in the 2nd sample when compared with other patients (p<0.01) (Figure 2B; Figure 3D). This group also included three patients (#9, 16, and 47) in whom *JAK2* Val617Phe was eliminated in 2nd sample (VAF <2,7%).

We also wanted to look closer at the three patients who endured to be treatment free from the time of diagnosis (#13, 23, 37). None of them experienced thrombotic complications or sMF. All of them were diagnosed with ET. Two of them had *JAK2* p.Val617Phe mutation with VAF on a relatively stable low level between the two measurements (range 8,13-11,8%). The third patient (#37) had *CALR* type 1 mutation with increasing VAF from 25,17% to 51,63% (Figure 2). Patients without treatment had a mean of 0.7 mutations detected in the second sample (one in two patients, none in one patient) compared to a mean of 1.7 mutations (range 0-5) in the rest of the group. Among non-driver mutations, those patients harbored *STAG2* p.Glu342Ter c.1024G>T with VAF of 3,08% (likely benign), *CBL* p.Asp460del c.1380_1382del with VAF of

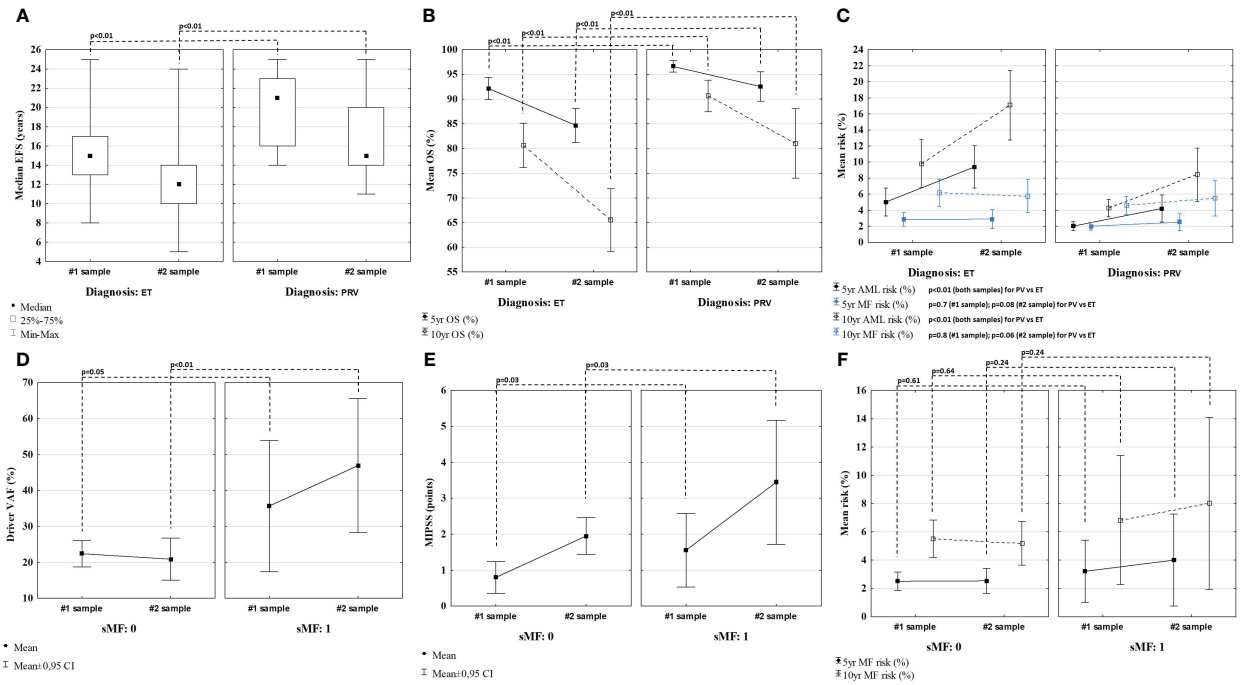

FIGURE 4
Comparison of event free survival (EFS) **(A)**, overall survival (OS) **(B)**, and 5- and 10-year risk of MF/AML **(C)**, calculated using MPN Personalized Risk Calculator using the data from the time of each sample collection. Comparison of driver mutation VAF **(D)**, score attributed by MIPSS **(E)** and MF risk **(F)** in patients with and without secondary myelofibrosis (sMF).

2,92% (no confirmed pathogenicity), *RUNX1* p.Arg423Gly c.1267C>G with VAF of 2,87% (no confirmed pathogenicity), *RUNX1* p.Glu422Ala c.1265A>C with VAF of 23,15% (benign) at 1st sample, and *DNMT3A* p.Gly109Ala c.326_327inv with VAF of 3,53% (undescribed), *DDX41* p.Val408Asp c.1223T>A with VAF of 4,10% (undescribed, detected frequently in this study), and *RUNX1* p.Glu422Ala c.1265A>C with VAF of 31,11% (benign) at 2nd.

3.4 Fibrotic progression

Seven ET (#2, 7, 10, 20, 39, 41 and 48) and two PV (#5 and 27) patients experienced fibrotic progression at follow-up. When compared to patients not developing fibrosis, patients with sMF had similar VAF of driver mutation at diagnosis (p=0.05) but significantly higher at follow-up (p<0.01) (Figures 4A, D). Patients with sMF had higher total points attributed to MIPSS, than those without fibrotic progression at diagnosis and follow-up (p=0.03 for both) (Figures 4B, E). Using MPN Risk Calculator and based on the data from the diagnosis, patients who later developed sMF during the disease had similar calculated 5- and 10-year MF risk (p=0.61 and p=0.64, respectively) as patients without fibrosis. Moreover, when the risk was assessed based on the results from the follow-up sample, when patients were in fact after or during fibrotic progression, the calculated 5- and 10-year risk of developing sMF was also comparable between patients with and without sMF (p=0.24 for both) (Figures 4C, F).

Since we identified a relatively high number of patients developing bone marrow fibrosis during our study, we wanted to investigate further specific variants detected in those patients. To assess the pathogenicity, we decided to distribute those findings into three groups – variants detected both at diagnosis and at follow-up, variants appearing in patients with sMF (not detectable at diagnosis) and variants detectable only at diagnosis (not detectable at follow-up) (Supplementary Table 2). The major findings in this group include the detection of *ASXL1* p.Gly646TrpfsTer12 (variant described in MF and connected with AML progression), *RUNX1* p.Leu56Ser and ZRSR2 p.Arg169Ter (variants described in MPN and MDS and connected with fibrotic progression) and U2AF1 p.Gln157Pro (described in MDS, MPN/MDS, MF and secondary AML).

3.5 Thrombosis

A total of six patients suffered from thrombosis during the time between each sample collection. We wanted to confront those findings with conventional risk assessment which is aimed at evaluating the risk of thrombotic complications. In ET, one patient from the high-risk group (without a history of thrombosis) and three of the non-high-risk patients (two below the age of 60, and one above the age of 60 at the time of thrombosis) experienced thrombotic complications (two ischemic strokes, one myocardial infarction and one pulmonary embolism). In PV, two high-risk patients (one had an ischemic stroke at diagnosis) and none of the low-risk patients experienced thrombotic complications. Of those patients with thrombotic complications, only one had a history of thrombosis.

Two patients diagnosed with ET and initially assessed as low- or very-low-risk, experienced thrombosis despite still being below 60 at the time of the second sample collection (#34 and 28). In the first patient (male ET) who suffered from myocardial infarction at follow-up, we detected *ZRSR2* p.Ser447_Arg448dup c.1338_1343dup variant at high VAF (ranging 84,78% - 84,38% between samples), which is suspected to increase the risk of thrombosis. In the second patient (female ET), who experienced multiple pulmonary embolism events during the disease, we observed increasing VAF of *JAK2* p.Val617Phe driver mutation, from 7,14% at diagnosis to 43,32% at follow-up, despite being on cytoreductive treatment with hydroxyurea, later in combination with anagrelide.

4 Discussion

In addition to known driver mutations, NGS-based studies allow for detecting numerous variants in various genes. Those findings must be considered with caution because not all variants are confirmed to be pathogenic. Since the molecular landscape of MPNs is undiscovered area, we performed a thorough literature search to look for associations of detected variants with pathogenicity. The two most frequently detected variants - *CBL* p.Asp460del and *RUNX1* p.Glu422Ala - were described in various neoplasms, including hematologic, with no confirmed pathogenicity (9, 10). Additionally, the total number of mutations detected in 2nd sample did not directly impact the disease's phenotype, indicating the need for qualitative rather than a quantitative approach. On the other hand, our analysis allowed us to detect significant molecular changes in patients otherwise labeled as TN – one with *TP53* p.Arg248Leu – a widely described, pathogenic hotspot gain of function mutation– and *RUNX1* p.Arg423Gly variant - undescribed previously, here associated with apparent features of MPN (11). Additionally, we found two coexisting driver mutations in two patients – *JAK2* p.Val617Phe along with *MPL* p.Trp515Leu and *JAK2* p.Val617Phe with non-canonical *MPL* p.Ser505Asn. In both cases *MPL* variants had higher VAF than *JAK2* p.Val617Phe, and the latter *MPL* variant is confirmed pathogenic (4, 12). In another patient, we found a non-canonical *JAK2* p.Lys607Asn variant co-occurring with *JAK2* p.Val617Phe, which was described in AML patients (13). In a routine workup done with the PCR method, identifying true driver mutation in such patients is challenging, underlying the importance of complete, thorough molecular evaluation in MPN patients in the future.

Unsurprisingly, the current study's two most frequently mutated genes were *TET2* and *DNMT3A*. Those mutations are widely described in hematologic neoplasms with inconsistent conclusions regarding their pathogenicity (14–17). Those and other mutations are also associated with clonal hematopoiesis of indeterminate potential (CHIP) - an effect of accumulation of specified mutations during life without a clear connection to morbidity (18). On the other hand, there are reports of increased

genetic instability in *TET2*- or *DNMT3A*-mutated patients, possibly triggering further clonal hematopoietic expansion and contributing to acquiring additional HMR mutations (19–23). By analyzing the dynamics of those mutations in our study, we show that *TET2*-mutated patients are bearing this mutation from the diagnosis and acquisition of *DNMT3A* occurs later during the disease. While we did not find the exact correlations with those mutations with clinical phenotype, we confirmed that *TET2* and *DNMT3A* mutations detected in the first sample resulted in a significantly more mutations detected in the second sample.

In PV and ET, conventional risk assessment is aimed at predicting the risk of thrombosis - one of the factors significantly influencing the mortality and morbidity of patients diagnosed with MPN (7, 8). Here, thrombotic complications occurring after diagnosis were recorded in six patients, with the majority classified as non-high-risk. In older patients, accumulation of cardiovascular risk factors, age and history of thrombosis are helpful variables for predicting the risk of thrombosis. However, in younger patients, otherwise classified as low- or very-low-risk, data from consecutive genetic analyses may help to predict thrombotic complications correctly. In our study, two young patients experienced thrombotic complications, despite not harboring conventionally accepted risk factors. One had a significantly increasing *JAK2* p.Val617Phe allele burden, a process which was proven to be associated with the risk of thrombosis by Soudet et al (24). The second patient harbored *ZRSR2* p.Ser447_Arg448dup variant, which is likely benign when detected in MDS, but connected with splanchnic vein thrombosis in MPN (25, 26).

Wider accessibility to modern diagnostic methods initiated the search for different variables predicting the outcomes of patients with MPNs. In 2019 Tefferi et al. proposed a new prognostication system, incorporating results from genetic analyses, for ET and PV - MIPSS-ET and MIPSS-PV, respectively (5). However, the authors acknowledge the need for further evaluation of these systems. In our study, we applied the MIPSS scoring system based on the results of two consecutive samples from each patient. In as many as five ET patients, detected mutations allowed for the increase in MIPSS score, placing those patients in the high-risk group and suggesting the possible utility of this system in assessing the risk in a dynamic fashion. Additionally, we show the utility of MIPSS-ET in predicting the risk of sMF. On the other hand, one young PV patient with a detected pathogenic *TP53* mutation remained in the low-risk group according to MIPSS-PV. The appearance of high-risk variants at follow-up underlines the utility of sequential molecular evaluation of patients with MPNs.

As an exploratory objective, we used the MPN Prognostic Calculator created based on the work of Grinfeld et al. (4). Authors underline that the tool is rather a proposition and is not yet validated. This analysis showed significantly better prognosis across all evaluated scores for patients with PV compared to ET. It failed to distinguish patients with a high risk of developing sMF. Whether this is the result of the construction of the tool itself or the generalization of input genetic data, remains to be further investigated. Regarding MPNs, we encourage reporting and analyzing the impact of specific variants rather than stratifying the risk by the presence of mutation only.

Fibrotic or AML progression remains the most therapeutically challenging dilemma in PV and ET patients. Here, nine patients developed sMF during their disease. None of those patients displayed features of fibrosis on the initial bone marrow sample. However, the evaluation of the characteristics of the megakaryocytes in those samples might not be in accordance with the current knowledge, hence it cannot be ruled out that a proportion of those patients had an evolution from prefibrotic to an overt stage of MF. Nevertheless, we revealed that patients with fibrotic progression had a significantly higher mutational burden of their driver mutation at samples collected during the transformation compared to baseline and to patients without sMF. Additionally, those patients harbored specific variants previously attributed to fibrosis, including *ASXL1* p.Gly646TrpfsTer12, *RUNX1* p.Leu56Ser, *ZRSR2* p.Arg169Ter and *U2AF1* p.Gln157Pro (4, 16, 27–33). Apart from the abovementioned variants, other mutations were detected only in those patients who developed fibrosis but their importance requires further investigation (Supplementary Table 2). While the sample size is relatively small, those findings support the idea of repeated monitoring, allowing for the detection of increasing VAF and pathogenic variants before fibrotic progression while being less unpleasant for the patient and more objective than trephine biopsy.

In PV and ET, the interest in the correlation between treatment and mutational landscape is growing. In our study, we performed analyses on two homogenous group of patients – those treated with HU monotherapy and patients that endured without any treatment during the study. Based on our results, the potential leukemogenic effect of prolonged exposure to HU remains questionable, since those patients did not present features of expanding mutational landscape. It is unclear whether this result is an effect of treatment efficacy or rather phenotypically stable disease, not requiring treatment change. In treatment-free patients, detecting non-pathogenic variants and stable VAFs of driver mutations correlated with genetically stable disease and clinical picture. The question remains whether their stable molecular profile was the phenotype of the disease itself or, that by not introducing the treatment we did not expose the malignant clone to further genetic instability (34, 35).

In conclusion, we emphasize the need for careful interpretation of molecular findings, particularly the assessment of the pathogenicity of specific variants. Extensive studies evaluating the molecular profile of patients and confronting it with the diseases' phenotype are critical. However, to date, only few studies evaluated the molecular findings in a dynamic fashion. Patients with MPNs must be evaluated more than once throughout their disease and it is insufficient to assess the risk only once, especially in young patients. Based on numerous studies across all fields of hematology, it is evident that diseases' phenotype is driven and modulated by various molecular changes in addition to basic factors, such as age and history of thrombosis. This field is open for discovery, and authors

of the current study believe that a larger portion of this knowledge remains unknown, including transcriptomics, epigenetic modifications, interaction with the microenvironment and paracrine activity of both malignant and non-malignant cells.

Data availability statement

The data can be accessed by the following link: https://datadryad.org/stash/share/MSNwkQvXlqHqOWyTvAiizUBZBS6UBJZaxqSqwLXtHaM.

Ethics statement

The studies involving humans were approved by Bioethics Committee for Scientific Research, Medical University of Gdansk. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

PS – conducted the research, recruited patients, analysed the data and written the manuscript. BW and MŻ – performed NGS analysis and reviewed the manuscript. AL – performed DNA isolation and reviewed the manuscript. MB – supervised the research, coordinated the work, helped writing and reviewed the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fonc.2023.1224590/full#supplementary-material

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XI. OŚWIADCZENIA WSPÓŁAUTORÓW

Gdańsk, dnia 4. 9. 2023

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Jako współautor pracy pt.

„Secondary chronic myeloid leukemia in a patient with CALR and ASXL1-mutated primary myelofibrosis"

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

wykonanie części analiz laboratoryjnych.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. **Patryka Sobieralskiego** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład lek. **Patryka Sobieralskiego** przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

Aleksandra Leszczyńska

(podpis współautora)

Gdańsk, dnia 04.09.2023

Prof. dr hab. Bartosz Wasąg
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„Secondary chronic myeloid leukemia in a patient with CALR and ASXL1-mutated primary myelofibrosis"

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analiza wariantów genetycznych

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Bartosz Wasąg

(podpis współautora)

Gdańsk, dnia 13.09.2023

Dr Monika Żuk
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„Secondary chronic myeloid leukemia in a patient with CALR and ASXL1-mutated primary myelofibrosis"

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:
analiza molekularna.

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. **Patryka Sobieralskiego** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

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Monika Żuk
.....

(podpis współautora)

Gdańsk, dnia 07.09 2022

Prof. dr hab. Jan Maciej Zaucha
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„Secondary chronic myeloid leukemia in a patient with CALR and ASXL1-mutated primary myelofibrosis"

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:
redakcja pracy, finalna jej wersja, współredakcja odpowiedzi recenzentom, korespondencja z edytorem

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. **Patryka Sobieralskiego** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

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Jan Zaucha

(podpis współautora)

Gdańsk, dnia 7.09.2023

Dr hab. Maria Bieniaszewska
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„Anagrelide in essential thrombocythemia: Efficacy and long-term consequences in young patient population"

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:
Koncepcja i projekt pracy, współudział w analizie danych, pisanie manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. **Patryka Sobieralskiego** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

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(podpis współautora)

Gdańsk, dnia 4.9.2023

Lek. Patryk Sobieralski
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„Anagrelide in essential thrombocythemia: Efficacy and long-term consequences in young patient population"

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

Przeprowadzenie analiz, napisanie manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. **Patryka Sobieralskiego** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład lek. **Patryka Sobieralskiego** przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

Sobieralski

(podpis współautora)

Gdańsk, dnia, 4.09.2023

Dr Aleksandra Leszczyńska
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„Anagrelide in essential thrombocythemia: Efficacy and long-term consequences in young patient population"

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

wykonanie części analiz laboratoryjnych

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. **Patryka Sobieralskiego** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

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Aleksandra Leszczyńska

(podpis współautora)

Gdańsk, dnia 06.05.2023

Dr Magdalena Dutka
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„Anagrelide in essential thrombocythemia: Efficacy and long-term consequences in young patient population"

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

pomoc w opracowaniu tekstu oraz poprawki

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. **Patryka Sobieralskiego** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład lek. **Patryka Sobieralskiego** przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

Magdalena Dutka

(podpis współautora)

Gdańsk, dnia 14.9.2023

Lek. Patryk Sobieralski
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„The molecular profile in patients with polycythemia vera and essential thrombocythemia is dynamic and correlates with disease's phenotype"

oświadczam, iż mój własny wkład merytoryczny w przygotowanie, przeprowadzenie i opracowanie badań oraz przedstawienie pracy w formie publikacji to:

Uzyskanie zgody komisji bioetycznej, uzyskanie finansowania projektu, identyfikacja grupy badanej, zebranie danych klinicznych i laboratoryjnych, przeprowadzenie analiz, napisanie manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. **Patryka Sobieralskiego** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, iż samodzielna i możliwa do wyodrębnienia część ww. pracy wykazuje indywidualny wkład lek. **Patryka Sobieralskiego** przy opracowywaniu koncepcji, wykonywaniu części eksperymentalnej, opracowaniu i interpretacji wyników tej pracy.

Sobieralski
.....

(podpis współautora)

Gdańsk, dnia 04.09.2023

Prof. dr hab. Bartosz Wasąg
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„The molecular profile in patients with polycythemia vera and essential thrombocythemia is dynamic and correlates with disease's phenotype"

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analiza wariantów genetycznych

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(podpis współautora)

Gdańsk, dnia 4.09.2023

Dr Aleksandra Leszczyńska
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„The molecular profile in patients with polycythemia vera and essential thrombocythemia is dynamic and correlates with disease's phenotype"

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Aleksandra Leszczyńska

(podpis współautora)

Gdańsk, dnia 13.09.2023

Dr Monika Żuk
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

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Monika Żuk
(podpis współautora)

Gdańsk, dnia 7.09.2023

Dr hab. Maria Bieniaszewska
(tytuł zawodowy, imię i nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt.

„The molecular profile in patients with polycythemia vera and essential thrombocythemia is dynamic and correlates with disease's phenotype"

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Współudział w tworzeniu projektu pracy, nadzór nad badaniem, weryfikacja manuskryptu

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. **Patryka Sobieralskiego** jako część rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

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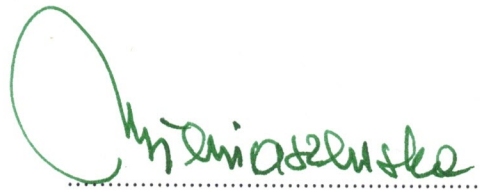

(podpis współautora)