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JAK2 INHIBITION IN MYELOPROLIFERATIVE NEOPLASMS

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Kandidaatintutkielmani tavoitteena on selvittää, mitkä asiat johtavat myeloproliferatiivisten neoplasmojen (MPN) puhkeamiseen, miten nykyään käytettävät hoitomuodot tehoavat niihin ja miltä myeloproliferatiivisia neoplasmoja sairastavien potilaiden tulevaisuus näyttää.

Tutkimusmenetelmänä oli teoreettinen kirjallisuuskatsaus. Kirjallisuuskatsaukseni aineisto koostui keskeisesti JAK-inhibiittoreja tutkivien kliinisten tutkimusten julkaistuista artikkeleista sekä myeloproliferatiivisen neoplasmojen (MPNs) tautiperimää käsittelevistä artikkeleista.

Kirjallisuuskatsauksen perusteella MPN:t voidaan karkeasti jaotella polycythemia veraan (PV), essentiaaliseen thrombosytemiaan ja primaariseen myelofibroosiin (PMF). Nämä alatyypit eroavat toisistaan tautikuviensa perusteella. Mutaatiot JAK2-, MPL- ja CALR-geeneissä johtavat häiriintyneeseen JAK/STAT-reittiin, mikä taas aiheuttaa liiallista verisolujen tuottoa.

Perinteiset hoitomuodot eivät ole riittäneet potilaiden tarpeisiin, joten uudenlaisia hoitomuotoja on tarvittu. Tähän tarpeeseen kehitettiin ensimmäinen FDA-hyväksytty JAK1/2-inhibiittori ruxolitinib, joka on parantanut potilaiden elämänlaatua. Tämä inhibiittori hoitaa kuitenkin vain oireita, mutta ei paranna itse tautia.

Ruxolitinibin jälkeen kaksi muutakin JAK2-inhibiittoria on saanut FDA-hyväksynnän: fedratinib ja pacritinib. Kehittämisvaiheessa on pyritty siihen, että ne olisivat ruxolitinibiä selektiivisempiä. Muutenkin niillä on hieman eriäviä ominaisuuksia kuin ruxolitinibillä, jotta sille intolerantit potilaat saisivat vaihtoehtoisen hoitomuodon. Fedratinib inhiboi JAK2:sen lisäksi BRD4:ä, ja pacritinib inhiboi JAK/STAT-reitin lisäksi TLR/Myddosome/IRAK1-reittiä, mikä antaa niille mahdollisuuden aiempaa tehokkaampaan ja turvallisempaan hoitoon.

FDA-hyväksyttyjen inhibiittorien lisäksi on lukuisia muita JAK-inhibiittoreita kehitteillä ja testattavana jo kliinisten kokeiden myöhäisessä vaiheessa. Koska JAK2-inhibiittoreiden käytöstä yksin saadut tulokset ovat riittämättömiä ja sivuoireet rajoittavat annostuksia, kiinnostus niiden käytöstä kombinaatioterapiassa muiden aineenvaihdunnallisesti aktiivisesti reagenssien kanssa on lisääntynyt. Kombinaatioterapioiden hyvät tulokset hoidon tehokkuudesta ja turvallisuudesta lisäävät myeloproliferatiivisista neoplasmoista kärsivien potilaiden toivoa avusta ja paremmasta tulevaisuudesta.

#myeloproliferatiiviset neoplasmat, #JAK2 inhibiittori, #Kombinaatioterapia

ABSTRACT

The method of my bachelor's thesis was a theoretical literature review. The aim of the literature review was to find out which are the causative agents for the development of myeloproliferative neoplasms (MPNs). How effective are the treatments used today and what does the future look like for patients with myeloproliferative neoplasms. My material mainly consisted of published articles of clinical trials investigating JAK inhibitors and articles dealing with the pathogenesis of myeloproliferative neoplasms (MPNs).

Based on my data and literature review, MPNs can be roughly divided into polycythaemia vera (PV), essential thrombocythemia, and primary myelofibrosis (PMF). These subtypes differ from each other based on their disease patterns. Mutations in the JAK2, MPL and CALR genes lead to a disrupted JAK/STAT pathway, which causes excessive production of blood cells.

Conventional treatment methods have not been sufficient for the patients' needs, so new types of treatment have been needed. The first FDA-approved JAK1/2 inhibitor, ruxolitinib, was developed to meet this need, and it has improved patients' quality of life. However, this inhibitor only treats the symptoms and does not cure the disease itself.

After ruxolitinib, two other JAK2 inhibitors have received FDA approval: fedratinib and pacritinib. During the development phase, efforts have been made to ensure that they are more selective than ruxolitinib and have slightly different properties than ruxolitinib, so that patients intolerant to ruxolitinib can receive an alternative form of treatment. In addition to JAK2, Fedratinib inhibits BRD4 and pacritinib inhibits the JAK/STAT pathway as well as the TLR/Myddosome/IRAK1 pathway, giving them the opportunity for more effective and safer treatment.

In addition to the FDA approved inhibitors, there are numerous other JAK inhibitors under development and being tested already in the late phase of clinical trials. Due to the insufficient results of JAK2 inhibitors when used alone and the dose limiting side effects, interest in combination therapy with other metabolically active reagents has increased. The good results of combination therapies in terms of treatment efficiency and safety increase the hope for a brighter future for patients suffering from myeloproliferative neoplasms.

#Myeloproliferative neoplasms, #JAK2 inhibitor, #Combination therapy

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1. INTRODUCTION

Myeloproliferative neoplasms (MPNs) is a group of blood cancers characterized by an overproduction of mature blood cells. Somatic mutations in the hematopoietic stem cells (HSCs) are the main cause for the onset of the disease. HSCs are a type of stem cell that are responsible for producing close to every myeloid cell and B and natural killer (NK) cells through the process of hematopoiesis.(Vainchenker and Kralovics, 2017)

The BCR-ABL a fusion gene is formed from a chromosomal translocation between the breakpoint cluster region (BCR) gene and the Abelson murine leukaemia viral oncogene homolog 1 (ABL1) gene. BCR-ABL fusion gene is considered a hallmark of chronic myeloid leukaemia (CML) and sometimes acute lymphoblastic leukaemia (ALL). The BCR-ABL-negative myeloproliferative neoplasms (MPNs) can be broadly classified into three subtypes: polycythaemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF). All subtypes are driven by the hyperactivation of Janus kinase 2 (JAK2) tyrosine kinase which is caused by the deregulation in the cytokine receptor/JAK2 pathway also known as JAK/STAT pathway. PV is characterized by erythrocytosis, which is the overproduction of red blood cells, resulting in blood thickening and an elevated likelihood of blood clot formation. Patients with PV may experience symptoms such as headaches, dizziness, and shortness of breath, and are at an increased risk of developing complications such as stroke or heart attack. ET is characterized as thrombocytosis in which the body produces too many platelets. Patients with ET may experience symptoms similar to a PV which makes it hard to distinguish between the diseases without a proper diagnostic. PMF is characterized by increased megakaryopoiesis and cytoses in the prefibrotic phase, progressive bone marrow fibrosis and cytopenia, meaning reduced blood cell count in more advanced stages which accumulates of fibrous tissue in the bone marrow. These can lead to anaemia, fatigue and weight loss, and are at an increased risk of developing complication such as infection. PMF carries the most significant symptoms that impair the patient's quality of life. All MPN subtypes can progress into myelofibrosis (MF) leading to the formation of fibrous scar tissue in the bone marrow. MF is a chronic bone marrow disorder characterized by progressive fibrosis, impaired blood cell production, and an enlarged spleen. Treatment of MF is mainly based on symptom burden and MF disease risk category rather than by the molecular profile or disease subtype.(Barbui *et al.*, 2018)

Recent advances in understanding the genetic and molecular basis of MPNs have led to improved diagnostics and treatment. The discovery of overactivated JAK2 signalling in every subtype increased the interest in JAK2 inhibition. Ruxolitinib the first JAK1/JAK2 inhibitor for the treatment of MF, developed more than a decade ago has provided symptom relief to patients. However, after many clinical trials, the observed side effects haven't been able to be prevented. In addition to side effects, ruxolitinib lacks the ability to reverse disease progression. At the moment MPNs are not curable and the patients with the disease are in desperate need of one. The possibilities of JAK2 inhibitors have been noticed in the treatment of MPNs and interest in them has exploded recently. The goal is to remove the side effects of already developed jak2 inhibitor treatments with modifying the specificity and function or combining them with other substances.(Leroy and Constantinescu, 2017)

This literature review aims to provide an in-depth analysis of the current state of research regarding JAK2 inhibitors in the treatment of MPNs. Specifically, this review will address the effectiveness of JAK2 inhibitors in the treatment of MPNs, and what are the potential improvement areas and limitations of these inhibitors.

2. MUTATIONS AS A CAUSATIVE AGENT OF MPNS

2.1 JAK2

Multiple somatic mutations have been found to cause initiation and disease progression of MPNs, but mutations in JAK2, MPL and calreticulin genes have been discovered to be the driving factors of myeloproliferative neoplasms. JAK2 gene encodes the Janus kinase 2 protein, which affects the pathways controlling cell growth, differentiation and survival through the JAK/STAT pathway. JAK2 is involved in cytokine receptor signalling and in haematopoiesis, immune responses and inflammation. As cytokines bind to their respective receptors, JAK2 activates phosphorylating downstream signalling molecules including STATs. Many different types of JAK2 mutation occur in the MPNs, with the mutations in the V617F and in the exon 12 being the most common. JAK2V617F mutation was discovered in 2005 and it is a somatic mutation where G is substituted into T at nucleotide 1849, in exon 14 of JAK2. This mutation substitutes valine into a phenylalanine at codon 617 in the pseudokinase domain. This domain inhibits the kinase domain and promotes cytokine-dependent activations meaning that the substitution of an amino acid causes gain-of-function causing the receptor to be cytokine-independent. This means that the receptor activates the STAT pathway even though cytokine hasn't bind to it and causing the hyperactivation of the JAK/STAT pathway. As the disease progresses the mutation changes from heterozygous to homozygous as a result of mitotic recombination.(McLornan, Percy and McMullin, 2006) Mutations in the exon 12 of JAK2 gene are present in the most cases of JAK2V617F- PV, meaning that the patient is diagnosed with PV without the presence of JAK2V617F mutation. Exon 12 mutation is usually only associated with PV but can progress into secondary myelofibrosis (MF). Mutations in the JAK2 exon 12 are located in the linker between the Src homology 2 (SH2) and the pseudokinase domains.(Passamonti *et al.*, 2011)

2.2 MPL

Thrombopoietin receptor (TPOR) is encoded by the MPL gene, missense mutations in this gene causes deregulation of the receptor. TPOR is responsible for the regulation of platelet production as it activates the JAK2 and STAT pathways, thus stimulating megakaryocyte growth and platelet production. This pathway doesn't have feedback regulation so the increase in platelet production due to mutation can't inhibit the pathway. Mutations occur in exon 10 with the most frequent being the tryptophan W515, located between the transmembrane and the cytosolic domains of MPL. This tryptophan as itself is responsible for the dimerization and activation of the TPOR, so mutations like W515L/K leads to self-activation of the receptor without the presence of thrombopoietin. Activation of the receptor also activates downstream signalling pathways which include JAK-STAT

pathway, PI3K-Akt pathway and Ras-MAPK pathway. Constitutive activation of the signalling pathways leads to abnormal proliferation and survival of hematopoietic stem and progenitor cells, leading to the development of ET and PMF.(Defour *et al.*, 2016)

2.3 CARL

Calreticulin protein affects the protein folding and quality control of N-glycosylated proteins in the endoplasmic reticulum. CALR frameshift mutations; insertions or deletions in exon 9 of the CALR gene, results in a novel C-terminus on the protein. There are two main types of CALR mutations accounting for 85% of all alterations. Type 1 mutations involve a 52-base pair deletion between c.1092 and 1143 and it results in the creation of a novel amino acid sequence at the C-terminus of the protein. Type 1 mutations account for about 44% to 53% of all CALR mutations. Type 2 mutations involve a 5-base pair insertion (TTGTC) between c.1154 and 1155 and result in a different novel amino acid sequence at the C-terminus of the protein in 32% to 42% of patients. Both types of mutations result the calreticulin protein to be misfolded and the hyperactivation of signalling pathways that control cell growth and proliferation.(Prins *et al.*, 2020)

Although mutations of the three genes reviewed earlier have been found to be the driving factors of MPN, so called triple negative MPN is a type of MPN where JAK2, MPL and CALR mutations do not occur with the disease. Therefore, all the driving mutations for MPNs haven't been found and causative agent for the triple negative MPN is still unknown. Albeit rare, two driving mutations can occur in the same patient. Patients with so called double hits, can also experience increased symptom burden, and a higher risk of disease progression. Additional somatic mutations in epigenetic regulation, splicing, signalling, transcriptional regulation and DNA repair can cause progression of the disease and aggravate the symptoms.(Leroy and Constantinescu, 2017)

Table 1. Driving mutations of MPN and their frequency in subtypes.(Rumi and Cazzola, 2017)

Mutation	Frequency (%)			Molecular Function
Gene	Location	PV	ET	PMF	
JAK2	V617F	95	50-60	50-60	Non-receptor tyrosine kinase mediating hematopoietic cytokine signalling
JAK2	Exon 12	2-3	-	-	
CARL	Exon 9	<1	26	18-32	ER chaperone protein interacting with thrombopoietin receptor MPL
MPL	Exon 10	<1	4	5-9	Thrombopoietin receptor

The severity of disease caused by MPNs, and the significance of symptoms depend on the variant allele frequency (VAF). A higher VAF is often linked to more severe symptoms, such as increased

spleen size, higher risk of thrombosis and with quicker and more significant disease progression. Lowering the VAF in MPNs is not a direct cure for the disease, but rather a therapeutic goal, potentially leading to improving patient's quality of life and a better overall prognosis.(Vainchenker and Kralovics, 2017)

3. CONVENTIONAL TREATMENT METHODS FOR MPNS

Currently conventional treatments are used to treat the disease based on the symptoms rather than the underlying causative agents. For example, for the treatment of anaemia related to MF supportive therapies such as erythropoiesis-stimulating agents (ESAs), Androgens, immunomodulating drugs, corticosteroids or splenectomy can be used to increase the haemoglobin levels in the patient. In the case of inadequate EPO levels, ESAs activate erythropoiesis and, consequently increasing EPO levels which improve patient well-being.

Splenomegaly, one of the characteristic features of myelofibrosis is due to increased splenic extramedullary haematopoiesis. Its treatment is usually only started when more symptoms appear, because the forms of treatment used today can worsen cytopenia. These therapies include cytoreductive drugs such as hydroxyurea or interferon-alpha to reduce the production of blood cells, particularly red blood cells or platelets. However, the use of this drug has its disadvantages, as it can increase the symptoms of anaemia, in which case anaemia-relieving drugs are needed as part of the treatment. In addition to this, mouth and foot ulcers are common when using hydroxyurea. And that's not all, because the majority of patients need alternative treatment in the longer term because they develop an intolerance to the medicine. As in the treatment of anaemia, splenectomy can also be used in the treatment of an enlarged spleen. However, it is a very risky procedure, with perioperative morbidity around 30% and mortality rate of 9%. Complications of the procedure include bleeding, infections and thrombosis, which is why it is used rarely in the treatment of anaemia and splenomegaly. Radiation therapy is also used as a form of treatment for patients who are unable to take medication or are not suitable candidates for surgery. Though it involves severe risks of prolonged cytopenia.(Cervantes, 2014)

Transplantation, currently the only curative treatment developed for the treatment of MPN, thus it’s only used for patients with high- and intermediate-2-risk MF. In the process, the patient's unhealthy bone marrow is replaced with the donor's healthy hematopoietic stem cells. When successful, the transplanted HSCs reverse the development of the disease and cure the disease, but the procedure involves many difficulties and risks. The biggest difficulty is finding a suitable stem cell donor. Risks include infection, rejection, and other complications during and after surgery. Conventional treatments still do not satisfy patients, so additional forms of treatment have had to be developed.(Cervantes, 2014) One of these is JAK inhibitors, which are discussed in the next chapter.

4. JAK2 INHIBITORS

As concluded earlier despite the mutation, all which subtypes exhibit an unregulated JAK-STAT pathway. This plays a key role in the pathogenesis of MPNs, leading to aberrant cellular proliferation and survival. JAK/STAT pathway is a crucial signalling pathway involved in regulating cell growth, differentiation, and immune responses. Pathway plays a central role in regulating gene expression and cellular processes in response to extracellular signals, particularly cytokines and growth factors. It consists of JAKs and signal transducers and activators of transcription (STATs). As specific ligands such as interferons and interleukins bind to the cytokine receptor, the receptor undergoes conformational changes that enable JAKs to phosphorylate. Phosphorylation of JAKs creates docking site to STATs as they phosphorylate the receptors in which STATs bind to. As STATs bind to their receptors, they are also phosphorylated and activated to the function of activating or repressing target gene transcription. STATs are not the only downstream signalling pathway of JAKs, as other pathways such as PI3K-AKT-mTOR-Forkhead transcription factors signalling proteins and Ras-Raf-MEK-ERK. AKT is essential for the differentiation process induced by EPO, whereas EPO-induced cellular proliferation relies on MAPK signalling.(Steelman *et al.*, 2004)

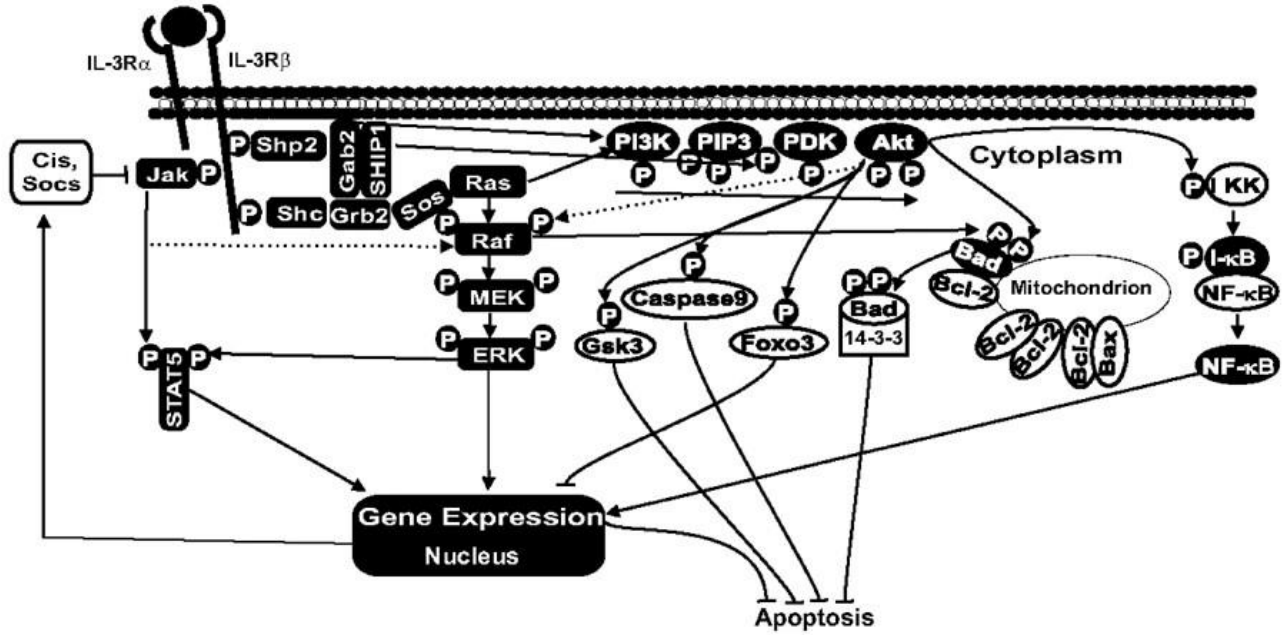

Figure 1. Reproduced with the permission of Copyright Clearance Center: Overview of effects of IL-3 on JAK/STAT, Raf/MEK/ERK and PI3K/Akt signaling.(Steelman et al.*, 2004)*

One approach to addressing this dysregulated signalling is the use of JAK2 inhibitors, which targets the JAK2 kinase that is frequently mutated and hyperactivated in MPNs. JAK2 inhibitors must have specificity for JAK2 so off-target toxicity can be avoided. Developed inhibitors cannot distinguish

between the mutated and wild type (WT) JAK2, so the amount of the dose must be regulated precisely, with too much inhibitor the normal function of JAK2 is inhibited too much resulting in disruption of normal metabolic pathways, or too little so that the inhibitor has no therapeutic effect. Ideally used inhibitor is able to recognize the mutated protein from the WT JAK2 in order to achieve complete mutated JAK2 inhibition.(Leroy and Constantinescu, 2017)

JAK2 inhibitors can be divided into two classes, type 1 and type 2 inhibitors. Type 1 inhibitors bind to the active conformation of JAK2 kinase domain and inhibit its activity by blocking the ATP-binding site. Unlike type 2 inhibitors type 1 inhibitors are already in use. These include ruxolitinib and fedratinib, both of which have been FDA approved for the treatment of MPNs. These drugs have demonstrated clinical efficacy in reducing spleen size, improving symptoms, and prolonging survival in patients with myelofibrosis. Type 1 inhibitors target mutated and non-mutated myeloid progenitors, but as of now there is no evidence that they are able to eliminate mutated hematopoietic stem cells (HSCs). These HSCs are disease-causing cells that must be eliminated in order to cure the disease. HSC renewal in patient is established by the persistent activation of STAT5, in order to be functional treatment for MPNs inhibitors must be able to distinguish between the mutated and the normal HSCs.(Leroy and Constantinescu, 2017)

4.1 Ruxolitinib

Ruxolitinib was the first targeted treatment developed for MPNs and after the COMFORT I and II clinical trials(Harrison *et al.*, 2012; Verstovsek *et al.*, 2012), approved for the treatment of myelofibrosis (MF) and PV patients with intolerance or resistance for hydroxyurea. Ruxolitinib works as a dual JAK1/JAK2 inhibitor and inhibits these enzymes in JAK/STAT pathway, thus controlling the blood cell production. Despite the promising results in the COMFORT trials ruxolitinib has limited effectiveness as certain patients do not respond and some becoming intolerant to the treatment. Ruxolitinib does not cure the disease, but it improves the quality of life of those suited for the treatment. However, in the RESPONSE trial(Vannucchi *et al.*, 2015), loss of response to ruxolitinib was noticed in some patients. This was due to the heterodimerization of JAK2 enzyme with JAK1 or TYK2. In recent study conducted by Steffen Koschmieder Et al.(Koschmieder *et al.*, 2023) investigated ruxolitinib's efficacy and safety for patients with newly diagnosed PV. In this study ruxolitinib reduced patients' symptom burden as measured pruritus and sweats scores reduced. However, the most significant thing in the study was the reduced JAK2V617F allele burden which corresponds to the information obtained from the RESPONSE study. 109 Adverse events were reported in 24/28 patients, but no significant side effects were reported and none of the patients stopped taking ruxolitinib because of these. Ruxolitinib has provided relief in MPN patients, as it alleviates its symptoms, but it has little to no impact on the JAK2 allele burden and bone marrow fibrosis, so it doesn't cure MF.(Harrison *et al.*, 2012; Verstovsek *et al.*, 2012; Vannucchi *et al.*, 2015)

4.2 Fedratinib

In recent years Fedratinib was developed for the treatment of MPNs. It has good JAK2 selectivity as it binds to the kinase domain at the ATP and peptide-substrate binding site. Fedratinib also has inhibitory effects on bromodomain-containing protein 4 (BRD4). The combination of inhibiting JAK/STAT pathway and BRD4 was demonstrated in animal models of MPNs. This treatment exhibited a synergistic effect by inhibiting NF-kB hyperactivation and reducing the production of inflammatory cytokines resulting in reduced symptom burden and reversal of bone marrow fibrosis. Regardless of the mutations causing the disease, fedratinib is effective at spleen reduction and alleviating symptom burden. Fedratinib as well as other developed inhibitors cannot distinguish between the wild type and mutated JAK2 resulting in clinical trials for the correct dosage as it is vital for the treatment to be effective without significant side effects. Patients with JAK2V617F mutation, fedratinib has shown differing results in allele burden alleviation. Phase 1 study showed reductions in JAK2V617F VAF were observed in patients with higher mutation burden. Unlike the phase I study, phase 2 study showed no consistent change in JAK2V617F allele burden when MF patients were treated with fedratinib doses of 300, 400 and 500 mg daily. Long-term effects of fedratinib have been studied in the NCT00724334 trial and effectiveness of long-term use of fedratinib was confirmed and the daily dosage of 400 mg was deemed to be most optimal. Fedratinib has provided alternative treatment for MF patients with limited therapeutic options. It has shown to be effective at reducing symptom burden of patients but like ruxolitinib, it lacks the ability to cure myelofibrosis.(Talpaz and Kiladjian, 2021)

4.3 Pacritinib

In 2022 pacritinib a JAK2/Fms-like tyrosine kinase 3 (FLT3) inhibitor got approved by the FDA for the treatment of MF and severe thrombocytopenia. As pacritinib is able to inhibit the JAK2 enzyme with high specificity and reduce myelosuppression, it also has inhibitory effects on the interleukin-1 receptor-associated kinase 1 (IRAK1) thus supressing the interleukin 1 directed inflammatory pathway. Pacritinib ability to inhibit JAK/STAT and TLR/Myddosome/IRAK1 pathways results in the suppression of NF-kB and downstream inflammatory cytokine cascade, leading to decrease in splenomegaly and effective management of MF symptoms. It can also inhibit BCRP, OCT1, OCT2, and P-glycoprotein transporters. Unlike dual JAK1/2 inhibitors ruxolitinib and momelotinib, pacritinib does not inhibit JAK1 which is known to impair megakaryopoiesis and, further, platelet production. Since parcitinib is not active against JAK1, it may contribute to its haematological stability. Future studies will delve deeper into evaluating the clinical benefits of pacritinib as a monotherapy or in combination with emerging therapeutic options.(Mascarenhas, 2022)

4.4 JAK inhibitors in late-stage clinical trials

There are multiple on-going clinical trials regarding JAK2 type 1 inhibitors. These trials include inhibitors such as ilginatinib, momelotinib, lestaurtinib and gandotinib. These inhibitors have the same principles that ruxolitinib and fedratinib has; to inhibit the JAK/STAT pathway to alleviate the symptom burden caused by the MPN. Momelotinib, a triple JAK1/2/ACVR1 inhibitor comparable to ruxolitinib, is able to reduce spleen size and general relief of symptoms. Unlike ruxolitinib it has unique ability to inhibit hyperactivated ACVR1/ALK2 signalling, which allows the hepcidin transcription to activate, increasing iron and haemoglobin levels in circulation. This stimulates erythropoiesis leading to alleviated symptoms of anaemia.(Mascarenhas, 2022) Ilginatinib, a JAK2 type 1 inhibitor, which has shown that it could possibly distinguish between the JAK2V617F mutated and the WT type JAK2 enzyme. However, different studies have shown divergent result regarding its selectivity. In the phase 1 study neurological side effects such as dizziness, peripheral neuropathy and headache were recorded at higher doses of ilginatinib. Ilginatinib is currently in the phase 2 which studies the efficacy and safety of it in patients with PMF, post-PV MF, post-ET MF and severe thrombocytopenia.(Nakaya *et al.*, 2014)

Lestaurtinib is a Fms-like tyrosine kinase 3 (FLT3) inhibitor and as in demonstrated in mouse models was able to inhibit the proliferation of JAK2V617F mutated cells. Most clinical trials with lestaurtinib were well tolerated with minor AEs occurring. These included nausea, vomiting, anorexia and diarrhoea. Phase 2 study with lestaurtinib in patients with PMF, post-PV MF or post-ET MF were conducted with 22 participants and the results showed reduction in spleen size and two became transfusion independent but no patient experienced improvements in bone marrow fibrosis.(Santos *et al.*, 2010, p. 2)

Gandotinib is a potent, highly selective and ATP-competitive inhibitor of JAK2 tyrosine kinase. Gandotinib was found to be more effective in inhibiting JAK2V617F-driven signalling and cell proliferation in Ba/F3 cells than compared to interleukin-3-stimulated WT type JAK2 mediated signalling and cell proliferation. Monotherapy of gandotinib was evaluated in phase 2 study conducted to patients with MPNs. Most frequent study-drug related AEs being anaemia, hyperuricemia, fatigue, diarrhoea and thrombocytopenia. Over 90% of PV and ET patients with JAK2V617F mutation responded to the treatment while patients without the mutation had overall response rate of 43.7% with ET and no recorded responses with MF.(Berdeja *et al.*, 2018)

JAK2 type 1 inhibitors are a diverse class of compounds that effectively target the JAK/STAT pathway, offering potential therapeutic benefits. Each inhibitor exhibits unique kinase-selectivity, but all of them block the ATP-binding pocket which consequently inhibits Jak2, leading to downstream suppression of the pathway. While these inhibitors show promise in treating MPNs, they also have their advantages and disadvantages. The advantages lie in their ability to selectively target JAK2, reducing off-target effects better than previous inhibitors. However, the drawbacks include potential

side effects and the risk of developing drug resistance. Even today, too many patients with myelofibrosis are intolerant to the treatment as they develop cytopenia during it or they experience too many adverse events. Further research and clinical trials are needed to fully explore the efficacy and safety profiles of JAK2 type 1 inhibitors and their potential for clinical applications.(Mascarenhas, 2022)

4.5 Type 2 inhibitors

Type 2 inhibitors, bind to the same ATP-binding pocket on the JAK2 kinase domain as type 1 inhibitors, but it can recognize the inactive conformation of the JAK2 kinase stabilizing it, preventing phosphorylation and downstream signalling. Type 2 inhibitors also bind to the extra “DFG-out”, which is a pocket created by the DFG phenylalanine's side chain being outside of the hydrophobic spine. This means that type 2 inhibitors bind to two different point of the kinase resulting in a more specific binding and selectivity than type 1 inhibitors. One example of a type 2 inhibitor is NVP-BBT594, which has shown promising preclinical results in MPN models as it is able to stabilize the inactive conformation of JAK2 resulting in an unphosphorylated activation loop. With the oxygen atom from its urea group, NVP-BBT594 binds to JAK2 DFG motifs D994 with hydrogen bond, ATP-binding site of JAK2 via its pyrimidine group and to the adjacent hydrophobic pocket 2 with the dihydroindole moiety. NVP-BBT594s pharmacokinetic properties are not suitable for in vivo use but it serves as an important example of potential type 2 inhibitors. Additionally, type 2 inhibitors may have the advantage of selectivity, as they target only the mutated JAK2 kinase, sparing the wild-type JAK2 that is necessary for normal hematopoiesis.(Leroy and Constantinescu, 2017)

NVP-CHZ868, type II JAK2 inhibitor developed to resolve inhibitor persistence from MPN cells that had developed it during the treatment with type 1 inhibitors and to accommodate better physico-chemical and pharmacokinetic properties than its predecessors. Its effects on the type 1 resistant cells were tested in the study conducted by Koppikar et al.(Koppikar *et al.*, 2012) Results showed exhibited selectivity for pathogenic JAK2 activation against the WT in V617F-mediated and MPL W515L-mediated activation. Though CHZ868 does not qualify as a clinical candidate, as it exhibits off-target activity for VEGF receptor tyrosine kinase in addition to many others. It acts as a first prototype of type II inhibitors, that can be used in preclinical studies to examine the effects of this kind of inhibition on the disease.(Meyer *et al.*, 2015)

While type 2 JAK2 inhibitors hold promise as a potential therapeutic strategy, it is essential to recognize that they are still in the experimental stages of development. The goal for type 2 JAK2 inhibitors is to achieve complete inhibition of mutated cells without any off-target inhibition to other pathways. Extensive preclinical studies and early-phase clinical trials with developed inhibitors, have demonstrated encouraging results in terms of their potential therapeutic benefits. Continued research, rigorous clinical trials, and regulatory approval processes are necessary steps to ascertain their efficacy, safety, and overall benefit to patients.

5. INHIBITOR PERSISTENCE AND ADVERSE EVENTS

On paper JAK2 inhibitors are a great solution for the treatment of MPNs as they have great potential benefits, but unfortunately AEs, such as an increased risk of infections, anaemia and thrombocytopenia can occur. The side effects ensue, as the inhibitors have off-target inhibitory affects, thus they can greatly affect the immune response and other signalling pathways. Developed inhibitors cannot recognize mutated molecules from non-mutated ones therefore, the amount of dosage is vital to achieve effective treatment and low side effects.(Tefferi *et al.*, 2022)

Additionally, some patients may not respond to JAK2 inhibitor therapy at all, some intolerance for the therapy, and some develop inhibitor persistence in the long-term treatment. Originally JAK2 inhibitor persistence was described in the study conducted by Koppikar et al.(Koppikar *et al.*, 2012) In the study high concentrations of type 1 JAK2 kinase inhibitors were added to growing MPN model cells until drug resistance occurred. This drug-resistant state was proved reversible as the cells regain their sensitivity when the inhibitor was removed. The conclusion of the study was that stabilization and the increased transcription of JAK2 affect the persistent growth of cells cultured with JAK2 inhibitors. The formation of heterodimeric complexes of JAK2 with other JAK family members, particularly JAK1 and TYK2, was associated with ruxolitinib persistence and reactivation of JAK2 signalling. The stabilization of JAK2 protein during ruxolitinib treatment was found to be due to the fact that JAK2 bound to ruxolitinib is not susceptible to dephosphorylation and degradation. These structural changes induced by ruxolitinib may affect the regulation of JAK2 protein expression and activity. Inhibition of JAK2 protein levels using HSP90 inhibitors was identified as a strategy to counteract deregulated JAK2 protein expression during JAK2 inhibitor persistence. The role of JAK2 in JAK2 inhibitor resistance was confirmed by the genetic removal of the protein in MPN mouse models, suggesting the requirement of JAK2 protein to maintain a drug-resistant state. Despite JAK2 being bound to ruxolitinib and expected to be inactive, JAK2-dependent signalling still occurs during JAK2 inhibitor persistence. Another reason for the development of inhibitory persistence was recently discovered due to a comprehensive analysis of the JAK2 signalling landscape. YBX1 is a nucleic acid-binding protein whose tasks include mRNA splicing and processing in addition to transcription. Jayavelu et al.(Jayavelu *et al.*, 2020) Recently reported that in MPN mouse model with genetic absence of YBX1 responded effectively to ruxolitinib treatment with molecular remissions occurring. As the results suggest the absence of YBX1 sensitizes cells to growth inhibition and apoptosis induced by JAK2 inhibitors, defining a potential genetic-drug synthetic lethality. YBX1 protein is located downstream of MEK/ERK pathway so in order to reduce YBX, this pathway must also be inhibited in the treatment. As the study suggest absence of YBX1 and its

downstream signalling molecules such as MAPK-interacting kinase 1 (MNK1), could lead to prevention of inhibitor persistency and to better efficacy of ruxolitinib treatment.(Koppikar *et al.*, 2012; Jayavelu *et al.*, 2020)

As new mechanisms for the inhibitor persistence are found, new ways to counter them are needed. There is ongoing research to improve the efficacy and safety of JAK2 inhibitors, as well as to identify biomarkers that can predict response and resistance. These studies include combination therapies as the evidence strongly supports the fact that MPN cells can persistently survive and proliferate during JAK2 inhibitor monotherapy. Combination therapies can be introduced in the beginning of treatment to prevent the development of inhibitor persistence or if inhibitory persistence has already developed, then specifically target the pathways responsible for maintaining persistence.

6. COMBINATION THERAPY WITH JAK2 INHIBITORS

Due to the lack of success with the reversion of disease progression and the development of inhibitor persistence with monotherapy JAK2 inhibitor, a major part of interest has shifted into combination therapy with other metabolically active factors. Combination therapies has primarily been focused on ruxolitinib-based combinations since it has been in the market for over a decade and other FDA approved JAK2 inhibitors fedratinib and parcitinib have been developed and approved quite recently. Combination therapy aims to alleviate symptoms that JAK2 inhibitors cannot influence, or to strengthen the effect of the inhibitor by making the target cells more sensitive to the JAK2 inhibitor or by preventing inhibitor persistence. With anaemia being the most frequent AE to occur related to the treatment of MPNs via JAK2 inhibitors supportive therapies such as ESAs and iron chelation therapy (ICT) which boost haematopoiesis, increasing haemoglobin levels have been tested in combination with ruxolitinib. Combination with ESAs received anaemia response in 54% of the patients while the response rate was slightly lesser with danazol (30%) and ICT (41%).(Kuykendall *et al.*, 2020)

6.1 IMiDs

Clinical trials combining immunomodulatory imide agents (IMiDs), including thalidomide and pomalidomide with ruxolitinib are currently on-going. Rationale for these studies is the fact that IMiDs alone have shown mild to moderate responses in improving anaemia, thrombocytopenia, and splenomegaly so combining them with a JAK2 inhibitor could provide better results than either one alone. Ruxolitinib combined with lenalidomide another IMiD, proved challenging due to AEs, particularly thrombocytopenia. Despite poor tolerance, some patients achieved a reduction in JAK2V617F mutant allele burden, which would indicate the possibility that IMiDs could be able to reverse the disease progression.(Kuykendall *et al.*, 2020)

6.2 TGFβ

Luspatercept and sotatercept are agents that act as activin receptor II ligand traps. They enhance the process of late-stage erythroblast differentiation by binding to ligands from the transforming growth factor-beta (TGFβ) superfamily, thereby reducing signalling through SMAD2 and SMAD3 pathways. TGFβ does play a critical role in the development of MF as it is involved in cell growth, differentiation and immune regulation. Both agents have showed the ability to achieve transfusion independence but with a somewhat low success rate due to the strict response criteria.(Kuykendall *et al.*, 2020)

Recently, the strategies of combination therapies have expanded beyond the off-label repurposing of drugs that have clinical profiles compatible with the effects of ruxolitinib. Thorough preclinical studies have identified specific targets that offer potential for synergistic or additive effects when combined with JAK2 inhibition. Additionally, these targets may also be able to restore a clinical response in patients who have experienced disease relapse or progression while receiving JAK2 inhibitor treatment.

6.3 PI3K

One of these approaches has to do with the PI3K pathway which is known to be dysregulated in MPNs and downstream of JAK2. PI3K pathway targets it downstream components AKT and mTOR, which represent therapeutic targets. The combination of ruxolitinib with dual PI3K/mTOR inhibitors showed enhanced efficacy in inhibiting proliferation and inducing apoptosis in MPN cells, surpassing the effects of each inhibitor alone. Additionally, this combination demonstrated potential for overcoming JAK2 inhibitor resistance. Clinical studies evaluating combination therapies involving PI3K inhibitors (such as umbralisib and buparlisib) with ruxolitinib in MF patients showed varying degrees of spleen reduction and clinical improvement, albeit with some adverse effects. Recent investigations with parsaclisib, a selective PI3K-delta inhibitor, in combination with ruxolitinib showed promising results in terms of spleen and symptom responses, with manageable safety profiles.(Kuykendall *et al.*, 2020)

6.4 HDACs

Histone deacetylases (HDACs) removes the acetylation of histone and nonhistone proteins thus controlling their activity. Acetylation affects transcriptional activation by opening the chromatin structure and thus enabling it to be transcribed. HDAC inhibitors have shown potential in preclinical and clinical studies for MPNs as they demonstrated efficacy by blocking MPN cell growth, inducing apoptosis, and sensitizing cells to JAK2 inhibition. Selective HDAC inhibitors are expected to improve therapeutic efficacy with fewer AEs. Pracinostat, a pan-HDAC inhibitor, was assessed in combination with ruxolitinib in two different phase 2 trials. First one of them showed potential results as the combination was able to reduce palpable spleen length more than half in 76% of the patients, but in the second combination proved challenging with dose interruptions and discontinuations due to hematologic toxicity. Another combination study with panobinostat and ruxolitinib showed promising early results but had varying outcomes in subsequent analyses. Improvements in selective HDAC inhibitors hold potential for effective MPN treatment with minimal AEs and the research continues to find HDACs compatible with JAK2 inhibitors.(Li, Rampal and Xiao, 2019; Kuykendall *et al.*, 2020)

6.5 Hedgehog Pathway Inhibitors

The hedgehog signalling pathway plays a role in haematopoiesis and has been implicated in MPNs. Increased expression of hedgehog signalling pathway target genes has been observed in MPN patients as well as in murine MF transplant model, suggesting its involvement in neoplastic phenotypes of MPN. In preclinical studies, combining a hedgehog pathway inhibitor, sonidegib, with JAK2 inhibition reduced leukocytes, platelets, mutant allele burden, and bone marrow fibrosis, highlighting it as a potential therapeutic target. Clinical trials combining hedgehog pathway inhibitors, sonidegib and vismodegib, with ruxolitinib in JAK inhibitor-naïve MF patients showed spleen and symptom responses similar to single-agent ruxolitinib, albeit with modest effects on mutant allele burden and bone marrow fibrosis. Patients' tolerability for the combination of the two inhibitors was a concern, with a high incidence of adverse events including increased blood creatine phosphokinase, thrombocytopenia and anaemia. Although preclinical study with a murine MF transplant model shoved evidence of the pathway being a valid therapeutic target, the effects on the model haven't been able to replicate in clinical trials. So, it remains to be seen whether it will be possible to eliminate the occurring AEs and make the treatment effective in clinical trials as well as it has been on the murine models.(Kuykendall *et al.*, 2020)

6.6 Bromodomain and extra-terminal (BET) inhibitors

BET inhibitors prevent proteins containing BET domains from binding to acylated histones and thus regulating the transcriptional activation of histones target genes. BET inhibitors prevent transcriptional activation of NF-kB target genes which are recognized as a pathologic factor in MPNs. Preclinical studies have shown BET inhibitors are able to inhibit MPN cell growth and induce apoptosis of targeted cell lines. BET inhibitors have shown synergistic responses with JAK2 inhibitors as well as preventing and overcoming JAK2 inhibitor resistance. In mouse models BET inhibitors were able to decrease the allele burden and reverse bone marrow fibrosis. BET inhibitor CPI-0610 is currently in clinical trial as multicohort study of MF patients including the combination with ruxolitinib. The goal for JAK2/BET inhibitors is to develop a single drug capable of targeting these critical proteins in MPNs.(Li, Rampal and Xiao, 2019; Kuykendall *et al.*, 2020)

6.7 DNA methyltransferase inhibition

MPN patients with JAK2 mutations exhibit abnormal DNA methylation patterns. Specifically, there is a decrease in the methylation of certain histone subsets due to increased binding of protein arginine methyltransferase 5 (PRMT5). This alteration leads to the stimulation of myeloproliferation. Preliminary results with the combination of hypomethylating agent azacitidine and ruxolitinib has shown promising results as patients response rate for palpable spleen length reduction and spleen size reduction was greater than with ruxolitinib monotherapy. Reversing the progression of the

disease was also noted with allele burden reduction with 87% of the assessable patients and reduction in bone marrow fibrosis with 41% of the patients.(Li, Rampal and Xiao, 2019; Kuykendall *et al.*, 2020)

6.8 Interferon alpha

Interferon alpha (IFN-α) has been a treatment option for MPNs for a long time because of its antiproliferative and immunomodulatory effect. IFN-α has disease-modifying potential as it is able to induce molecular remissions by normalizing blood cell counts and reducing the burden of mutated cells. It can also improve symptoms associated with MPNs and potentially delay disease progression. The use of IFN-α is limited by its toxicity as it has the ability to affect critical immune responses. Combination of pegylated (PEG) forms of IFN-α and ruxolitinib has been studied in the clinical trial NCT02742324, which tried to identify the optimal combination dose for PEG-IFNa2 and ruxolitinib was completed and final results show that the combination of selective targeting by mutated progenitors provided high rates of reductions in spleen length and JAK2V617F allele burden.(Silver, 2020; Kiladjian *et al.*, 2022)

6.9 Poly-ADP-ribose polymerases (PARPs)

MPN cells exhibit increased levels of reactive oxygen species (ROS) and stalled replication forks, leading to the accumulation of harmful DNA double-strand breaks (DSBs). The survival of MPN cells rely on their ability to repair these DSBs. Poly-ADP-ribose polymerase 1 (PARP1) is a crucial player in preventing and repairing these lethal DSBs. PARP1 activates base excision repair and single-stranded DNA break repair, stimulates fork repair/restart, and facilitates the backup repair known as non-homologous end-joining (B-NHEJ). The presence of potentially lethal DSBs in MPN cells presents an opportunity to selectively target and eliminate these cells by disrupting DNA repair mechanisms. Ruxolitinib was found to inhibit BRCA-mediated homologous recombination and DNA-dependent protein kinase-mediated nonhomologous end-joining which are two major DSB repair mechanisms.(Kleppe *et al.*, 2018) Ruxolitinib combined with olaparib a PARP inhibitor, toxic DSBs accumulated in MPN primary cells causing enhanced elimination of these cells. More importantly, the combination of PARP inhibitor talazoparib, ruxolitinib and hydroxyurea showed high effectivity against JAK2V617F, CALR (del52), and MPLW515L primary MPN xenografts.(Li, Rampal and Xiao, 2019)

The field of combination therapies with JAK2 inhibitors are blooming at the moment. These examples are just the tip of the iceberg in this field and as more results from on-going trials emerge and the knowledge of MPNs improves, the effectiveness and safety of treatments increases. Despite the challenges with comparing the results of phase 2 trials, several agents including PCPI-0610 (NCT04603495), a BET inhibitor and navitoclax (NCT04472598), a BC-XL inhibitor are already being planned and active for phase 3 combination studies. These phase 3 studies will provide

comparative data to determine the effectiveness of combination approaches compared to mono-therapy with JAK2 inhibitors and conventional treatment options.

7. FUTURE DEVELOPMENTS

With the increasing interest in JAK inhibitors and a growing understanding of their limitations, it is crucial to explore future developments. Dose-limiting adverse events caused by the inhibition of JAK2-mediated growth factor receptor signalling have prompted the search for alternative options. One potential approach is to identify allosteric binding sites in JAK2. For example, the druggable allosteric cysteine in the non-catalytic pseudokinase domain of JAK1 (C817) and TYK2 (C838) represents such a binding site. Binding electrophilic compounds to the JAK1 site blocks JAK1-dependent transphosphorylation and cytokine signalling. Allosteric and covalent inhibitors hold promise as potential alternatives for next-generation treatments. Similar binding sites may exist in JAK2, opening the possibility of developing JAK2 inhibitors with unprecedented specificity.(Kavanagh *et al.*, 2022)

A covalent inhibitor for JAK3, ritlecitinib, has been developed to target a cysteine (C909) in the activation loop of JAK3. Although this covalent inhibitor exhibits greater specificity than traditional JAK inhibitors, its target is not specific enough, as it also reacts with the TEC family kinases due to the shared cysteine with JAK3. Recently, the FERM-SH2 domains structure of JAK2 was solved, revealing potential receptor-binding sites for allosteric inhibitors. This discovery could pave the way for the development of more specific receptor engagement.(Leroy and Constantinescu, 2017; Kavanagh *et al.*, 2022)

Today, computer modelling can be used to identify suitable small-molecule fragments capable of binding to a specific binding site. Finding these ligandable and functional allosteric sites is the challenge, as they tend to be more diverse and less conserved than active sites. Advancements in computer modelling enable the potential of these allosteric and covalent inhibitors to be utilized by designing and optimizing their structures, targeting specific disease-causing proteins. By exploiting the unique properties of allosteric binding sites, highly potent and selective inhibitors can be developed, exhibiting improved efficacy and reduced off-target effects. The integration of computational approaches with experimental validation holds tremendous promise for the future of MPN treatment, holding the potential for more personalized and effective therapies.(Kavanagh *et al.*, 2022)

8. CONCLUSIONS

Interest of JAK2 inhibitors in treating MPNs, has soared in recent years. At the moment both monotherapy of JAK2 inhibitors and combination therapy with other metabolically active agents are considered as a treatment option for MPNs and from them to advanced myelofibrosis. MPNs are hematological disorders characterized by abnormal blood cell proliferation, primarily affecting the myeloid lineage. Disease-driving mutations in JAK2, MPL, and CALR genes have been identified as potential therapeutic targets.

JAK2 inhibitors, including FDA-approved ruxolitinib and fedratinib, have emerged as effective treatments for MPNs, particularly in myelofibrosis and polycythaemia vera patients. These inhibitors specifically target the dysregulated JAK-STAT signalling pathway implicated in MPN pathogenesis.

Clinical trials experimenting with JAK2 inhibitors consistently report improvements in key disease-related symptoms, such as splenomegaly, constitutional symptoms, and pruritus. Additionally, these inhibitors significantly reduce spleen size and improve patients' quality of life. Studies have also indicated favourable effects on overall survival and progression-free survival.

However, limitations and challenges associated with JAK2 inhibitor therapy were identified in this review. Adverse effects, including anaemia, thrombocytopenia, and infections, require careful monitoring and management. The development of inhibitor persistence in monotherapy of JAK2 inhibitors highlights the need for ongoing research and exploration of combination therapies to overcome this limitation.

Current research in JAK2 inhibitor therapy has shifted towards identifying new combinations, aiming to address inhibitor persistence and enhance therapeutic efficacy. Combinations are now focused on preventing persistence and breakthrough the limitations of JAK2 inhibitor monotherapy. Individuals suffering from MPNs are currently facing a critical demand for viable treatment alternatives. Currently, there is a considerable cause for optimism, given the significant advancements in understanding the disease's fundamental causes, the initiation of its onset, the trajectory of its progression, and the determination of symptom severity. These developments have substantially enhanced the prospects for effective therapies, instilling a renewed sense of hope.

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