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THE RATIONALE OF METFORMIN ON ESSENTIAL THROMBOCYTHEMIA

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Cancer, Platelets, Essential Thrombocythemia, Metformin.

ABSTRACT

Introduction: Essential Thrombocythemia (ET) is characterized by an increase of circulating platelets resulting from the proliferation of megakaryocytes in the bone marrow (BM), with risks of thrombosis and bleeding. Metformin might reduce neoplastic cell proliferation and reduces cardiovascular events in patients with diabetes. Objective: To evaluate the changes in peripheral blood platelets, Ki-67, and CD34 in bone marrow with the use of metformin in ET before the intervention (PI), after three months (P3), and after six months (P6). Method: Phase II, open-label study, with initial results of investigation of the clinical activity of metformin. Results: Pre-intervention blood count (PI), after three months (P3) and after six months (P6). Two of the four patients reduced the number of platelets in P6 compared to PI. The immunohistochemistry of bone marrow biopsy of the patient 1 CD34 cells 16 cells/mm² PI, 1 cell/mm² in P3, and 2 cells/mm² in P6. Ki67 proliferative index of 740 cells/mm² after six months. Patient 2 has CD34 cells, 4 cells/mm² in PI, 1 cell/mm² in P3, and 7 cells/mm² in P6. Ki67 in P6 35 cells/mm². Patient 3 CD34 1 cell/mm² in PI, 3 cells/mm² in P3. Ki67 140 cells/mm² in P3. Patient 4, CD-34 2 cells/mm² in PI, 1 cell/mm² in P3, and 2 cells/mm² in P6. The Ki67 260 cells/mm² in the P6. Conclusion: Metformin, with potential anti-inflammatory and anticancer functions, is important to be used in association with or even in the initial treatment of low-risk ET populations.

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INTRODUCTION

Myeloproliferative neoplasms (MPN) are chronic inflammatory conditions characterized by the mutation of cells that populate the bone marrow, hematopoietic cells, which have significantly increased (1). Three significant diseases constitute MPN, Polycythemia vera (PV), Myelofibrosis (MF), and Essential Thrombocythemia (ET). Studies suggest that NMP can be described as the "Human Inflammation Model" due to consequences such as premature atherosclerosis, immune system dysregulation, leukemia, and increased risk of cancer recurrence (2–4).

ET is a disease characterized by an increase in circulating platelets that result from the multiplication of bone marrow cells, the megakaryocytes (5); as complications of the disease, vascular involvement and acute myeloid leukemia occur (6). According to Navarro et al. (2016), thrombotic events impact the survival of patients with ET. The risk of thrombosis in these patients must be considered, in addition to the previous history of the disease being an established risk factor.

Pharmacological therapy in patients with ET aims to prevent vascular events (8). From this perspective, evaluating pharmacological strategies that can act on the risks of the disease and that present low therapeutic cost becomes relevant. Metformin is a drug used in patients with type 2 Diabetes Mellitus, which is related to vascular physiological improvement and reduces cardiovascular events (9,10). Clinical studies already point to the use of metformin in patients with leukemia, lymphoma, and multiple myeloma (11–15).

One of the pharmacological alternatives for patients with MPN is hydroxyurea, with potential long-term toxic effects (6). Therefore, low-cost drugs for treating diseases such as ET are necessary; likewise, metformin already shows evidence of its possible therapeutic use in neoplasms, whether alone or associated with other drugs in cancer treatment (9,10,16). ,17). In a preclinical study, Liang et al. (2017) investigated the action of metformin in myeloid leukemia; they found that the drug was active in inhibiting cell proliferation and inducing apoptosis of megakaryoblast cells. Therefore, evaluating the rational effect of metformin in myeloproliferative diseases becomes necessary (Figure 1).

Metformin reduces cell viability, proliferation, cell cycle progression, and clonogenicity in JAK2V617F positive myeloproliferative neoplasm (MPN) cells. In an exploratory study, new molecular mechanisms of action of metformin, apart and in combination with ruxolitinib, were established on aberrant JAK2V617F signaling and provided insights for developing alternative, complementary, or both therapeutic strategies for MPN (19).

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Essential Thrombocythemia and Vascular Changes:

ET is a chronic disease characterized by the proliferation of megakaryocytes, increasing the number of platelets (20). Patients are usually asymptomatic but may present vascular complications such as vasomotor alterations, hemorrhage, and thrombosis (7,8,21). The disease diagnosis is characterized by the laboratory identification of cellular hyperplasia and a constant increase in the total platelet count (22). Associated with the detection of JAK2V617F, CALR or MPL mutations confirm the presence of an underlying MPN. Still, its absence does not include the possibility since up to 20% of TE patients can be triple-negative (23). The recommended therapeutic approach is linked to normalizing the number of platelets, which seems to be related to preventing vascular events.

Legrand et al. (2021) analyzed two cases of patients with vascular complications associated with ET. In both cases, microvascular lesions and thrombosis were observed, which suggests that ET, due to its pathophysiology, can lead to the formation of thrombi that can trigger distal embolism. Thus, understanding the disease helps in the prognosis of these patients.

Metformin And Vascular Metabolic Control:

Metformin is a biguanide-derived drug that has been used in the treatment of type 2 diabetes mellitus (DM2). The drug action is related to glucose reduction due to metabolic regulation such as inhibition of hepatic glycogenolysis, reduced glucose absorption, and increased glucose uptake in peripheral tissues (10,17). The use of the drug for endocrine management is also related to platelet metabolic control.

Dolasik et al. (2013) verified the effects of metformin on the volume of platelets in patients with T2DM; and concluded that the use of the drug significantly reduced the volume of these cells. In addition, the authors infer that the use of the drug has positive effects on vascular adhesion molecules. Metformin also reduces platelet aggregation (9,26) and, as consequence, prevents thrombus formation.

The use of metformin has been studied due to its potential contribution to anticancer activity. Liang et al. (2017) investigated the effects of metformin on megakaryocyte apoptosis. Their findings clarify that the drug significantly inhibited the proliferation and apoptosis of platelet precursor cells. Thus, metformin is a potential therapeutic target in patients with ET.

CD34 AND Ki67:

Bone marrow derived CD34 POSITIVE cells are found in peripheral blood, are more elongated, and are active in angiogenesis (27). This protein is expressed by hematopoietic cells in various mature cells, such as mesenchymal and muscle satellite cells. Ki67 protein is also used as a cell proliferation

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marker. Its function is related to the interphase and mitotic cells, and its cellular disposition changes according to the cell cycle progression. Ki67 is also a marker used to classify different types of cancer (28). Thus, quantifying this protein is necessary to follow the regression or progression of the disease.

Tumors with low proliferation, response to chemotherapy, and a lower expression of Ki-67 are associated with a good prognosis. On the other hand, highly proliferative tumors are more sensitive to chemotherapy and have a high rate of Ki-67, have a predictive value of response to cytotoxic treatment. However, highly proliferative chemotherapy-resistant tumors and a high Ki-67 rate are related to poor therapeutic outcomes and lower survival (29).

Metformin And Cancer Cell Autophagy:

The effects of metformin are related to the inhibition of the inflammatory response and the growth of cancer cells. It acts on energy metabolism and prevents the proliferation of cells affected by the neoplastic process (18,30). With metabolic regulation, the drug induces apoptosis and directly interferes with the cell cycle (10,11). These metformin-mediated events may contribute to anticancer effects.

Metformin inhibits the mitochondrial respiratory chain, decreases ATP synthesis, and stimulates adenosine monophosphate-activated protein kinase (AMPK). This protein regulates the expression of cell cycle metabolic enzymes via p53 activation (10,17,31). The effects related to the activation mechanism of AMPK, responsible for cellular energy homeostasis, suppress the proliferation and aggressiveness of cancer (30,32). Thus, the drug has beneficial effects on cellular cascades, an essential factor regarding cancer and its long-term mediation.

The activity of metformin under the cellular respiratory chain and the cell cycle phases, which are crucial for cancer development, denote the potential pharmacological therapeutic use in patients with neoplasms. In general, metformin acts in the specific inhibition of the mitochondrial complex, and this function protects the mitochondria and reduces platelet activity on it, called hyperpolarization. This protection also prevents damage to the cell membrane and reactive oxygen species (9,25,30,31).

MATERIALS AND METHODS

Phase II, open-label study, with initial results of investigation of the clinical activity of metformin in patients with Essential Thrombocythemia in follow-up at Hospital do Câncer de Cascavel – UOPECCAN. This study aims to assess the overall response rate to metformin treatment in ET, platelet count less than or equal to 400x109 L, and resolution of palpable splenomegaly for at least 12 weeks. For patients to be considered responders, all criteria must be met during treatment (not necessarily for the same 12 weeks). Dose reduction of hydroxyurea use at week 12 will be

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considered a primary endpoint in those patients using the drug. Moreover, we assessed Ki-67 and CD34 in bone marrow with the use of metformin in ET before the intervention (PI), after three months (P3), and after six months (P6).

According to the World Health Organization (WHO), the diagnostic criterion for ET has major and minor criteria. The diagnosis of Essential Thrombocythemia requires the fulfillment of the four major or the first three major and one minor criterion.

Major Criteria:

1) Platelet count $\geq 450 \times 10^9$ /L.
2) Bone marrow with a proliferation of megakaryocytes with large and mature morphology. No significant deviation of granulopoiesis or neutrophilic erythropoiesis and very rarely increase (grade 1) of reticulin fibers.
3) Not meeting WHO criteria for CML, CSF, LM, MDS, ~~or other myeloid neoplasms.~~
4) Presence of JAK2, CALR or MPL mutation.

Minor Criteria:

1) Presence of a clonal marker (e.g., altered karyotype).
2) Absence of evidence of reactive thrombocytosis (through PCR, ESR, and ferritin tests).

In this study, we followed a two-stage experimental design by Simon (33); the project will be developed in two stages. The total population for this study will be 27 patients diagnosed with TE, with 13 patients being treated in the first stage. If no patient responds in the first stage, the study will be stopped early for futility. If three patients or fewer respond by the end of stage two (27 patients), then no further drug investigation is warranted.

After signing the Informed consent form, everyone will be submitted to pharmacological therapy with metformin. Before starting treatment, the researchers obtained samples of bone marrow for evaluation of the disease and blood for platelet counts. The samples will be collected before the pharmacological approach and after 3 and 6 months of treatment. Partial results from 4 patients who entered the study will be presented in this article.

On day 1, patients started using metformin (500 mg per day with the evening meal), increasing the dose gradually from 500 mg per week to a maximum of 2.5 g/day, excluding adverse effects greater than or equivalent to grade 2. Metformin was added to the patient's treatment regimen as an adjuvant drug. Participants did not fail to receive standard treatment for ET (hydroxyurea, anagrelide, aspirin, or interferon).

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Patients were asked to take their metformin tablets once or twice a day at approximately the same time each day and instructed to swallow the tablets with a full glass of water, not chew them. The study was conducted by the Declaration of Helsinki and the International Conference on Harmonization Guidelines for Good Clinical Practice.

RESULTS

The evaluated patients were two females and two males with a mean age of 56 years using the drugs Hydroxyurea and Anagrelide. In the laboratory test Hemogram, the following variables were evaluated: hemoglobin (g/dL), leukocytes (mm³), and platelets (mm³) before intervention (PI), after three months (P3), and after six months (P6). Patient 1 has PI hemoglobin of 13.74 g/dL, 12.76 g/dL in P3, and 12.86 g/dL in P6. Leukocytes were 7,09 × 109 /L in PI, 5,38 mm³ in P3, and 7.31 mm³ in P6. Platelets 759,5 × 109 /L in PI, 625,700 mm³ in P3, and 697,700 mm³ in P6. There was a decline in platelet numbers after six months of intervention.

Patient 2, hemoglobin at PI 13.63 g/dL, 13.74 g/dL at P3, and 13.56 g/dL at P6. Leukocytes were 5,9 × 109 /L in PI, 7,65 × 109 /L in P3, and 6.61× 109 /L in P6. In the analysis of PI platelets, 645,4× 109 /L was found in P3, 725,2× 109 /L, and 703.4× 109 /L in P6.

Patient 3 had PI hemoglobin of 14.86 g/dL, P3 15.70 g/dL, and P6 15.8 g/dL. In the leukocyte count in IP, 4,16 × 109 /L, 4,8 × 109 /L in P3, and 4,4 × 109 /L in P6. In the pre-intervention evaluation, the platelets were 302,4× 109 /L, in P3 575 × 109 /Land in P6 360× 109 /L³. In the variable number of platelets, the patient had a lower number of platelets in the evaluation after six months compared to 3 months of treatment.

In the analysis of the laboratory variables of patient 4, a hemoglobin of 15.1 g/dL, 13.28 g/dL in P3, and 13.10 g/dL in P6 was verified in the PI. Leukocytes of 6.30 mm³ in PI, 5,98 × 109 /L in P3, and 6,77× 109 /Lin P6. Platelets of 303 × 109 /L in PI, 307,1 × 109 /L in P3, and 316,6 × 109 /L mm³ in P6.

In this initial sample of the study, a bone marrow biopsy was performed, and the degree of fibrosis was evaluated. Three evaluations were performed, and the results observed demonstrate maintenance of the fibrosis degree. One patient had a grade of 2 during the study evaluations, and the other patients had a grade of 0.

The immunohistochemistry of the sample collected in the bone marrow biopsy was evaluated. Patient 1 shows the presence of CD34-POSITIVE cells at 16 cells/mm² in the biopsy before drug intervention, 1 cell/mm² after three months of treatment, and 2 cells/mm² in the 6-month biopsy with the use of metformin. Thus, there was a reduction in CD34 cells after drug intervention. The Ki67

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proliferative index was 350 cells/mm² before the intervention, 128 cells/mm² at three months, and 740 cells/mm² after six months of drug use. There was a reduction in CD34 cells after six months of treatment and an initial 3-month reduction in the Ki67 proliferative index.

Patient 2 has CD34-POSITIVE cells 4 cells/mm² in PI, 1 cell/mm² in P3, and 7 cells/mm² in P6. In evaluating Ki67 in PI, 180 cells/mm², P3 75 cells/mm² and P6 35 cells/mm². During the treatment, it was found that there was an increase in the expression of CD34 cells. Thus, the proliferative index of Ki67 was reduced after six months of treatment with metformin to the evaluation prior to the use of the drug.

Patient 3 showed CD34-POSITIVE cells 1 cell/mm² in PI, and 3 cells/mm² in P3. Ki67 in was 50 cells/mm² in IP and 140 cells/mm² in P3. Patient 3's 6-month biopsy was not collected due to the impossibility of attending the service.

In the evaluation of patient 4, POSITIVE CD-34 cells were found: 2 cells/mm² in PI, 1 cell/mm² in P3, and 2 cells/mm² in P6. The Ki67 in the PI of 360 cells/mm², 370 cells/mm² in the P3, and 260 cells/mm² in the P6. The expression of CD34 cells was constant during the 6-month treatment, and the proliferative index was reduced compared to the pre-intervention assessment.

DISCUSSION

In this partial study result, we can observe that metformin seems to have contributed to the reduction of Ki67 expression in 2 of the four patients evaluated. In 3 patients, it decreased the number of platelets or maintained a reduced dose of hydroxyurea or anagrelide. It was seen that one patient showed a significant initial reduction of Ki67.

Patients with ET have a high rate of Ki67 proliferative index when compared to control groups without the disease (34). Metformin is associated with protein kinase activation via cyclic AMP, a critical tumor suppression pathway. In addition, the use of the drug is associated with a lower risk of cancer in diabetic patients (17). Two of the four patients showed a reduction in Ki67 after a 6-month intervention with metformin.

The evaluation of CD34 cells in the present study showed a reduction in two of the four patients evaluated, and the others kept the number of cells constant. Metformin use does not affect normal CD34-POSITIVE cells, as it interferes with proliferation and induces apoptosis in human immortalized cells (17). In addition, metformin induces dephosphorylation of the translational regulator, 4E-BP1, which inhibits the initiation of messenger RNA translation. Thus, it reduces the recruitment of mRNAs that encode oncogenic proteins, which maintains normal hematopoiesis (35). This drug action may be related to the maintenance of CD34 cells in the present study.

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Patients with ET with microvascular alterations present increased b-thromboglobulin, platelet factor 4, high levels of thrombomodulin, and increased excretion of thromboxane B2, which indicates thrombotic processes that may be mediated by platelets (36). Treatment with metformin reduces platelet activation and has an anti-atherosclerotic impact (25). The present study found that patients had a reduction in the number of platelets after six months of continuous use of metformin.

The diagnosis of ET is based on bone marrow biopsy and the presence of major and minor criteria, of which fibrosis is a predictor factor (22). In this study, the degree of fibrosis in the bone marrow biopsy was evaluated, and the sample remained constant regarding the evaluated degree. However, patient 1 with grade 2 fibrosis using anagrelide had a platelet reduction with metformin that he had not previously had on hydroxyurea. Several studies reviewed patients previously classified as TE and stratified a considerable proportion of patients as pre-MF (180/1071: 16.8%) (37).

A phase II study (FIBROMET) showed that metformin is safe and well tolerated and that bone marrow fibrosis was reduced (38). However, the results were not statistically significant due to the small sample size. Indeed, Chronic inflammatory states may be involved in the initiation and progression of PMN, especially with fibrosis; thus, as IFN alpha and JAK2 inhibitors reduce the high levels of inflammatory cytokines (39,40), so does metformin (41), which may be responsible for the onset and progression of the disease, therefore presenting a solid reason for combination drug classes, decreasing factors are known to contribute to fibrosis and bone formation.

Immunohistochemistry is frequently used in oncology to identify tumor proliferation. The Ki67 expression rate is higher in patients with ET. In a biopsy, Ki67 cells are used as a prognosis and a predictor of cancer treatment's benefits or harms (42). In the patients evaluated in stage 1, it was found that there was an essential initial reduction of Ki67 compared to the pre-intervention amount.

Bai and Shao (2004) investigated apoptosis and bone marrow cell proliferation in patients with Polycythemia vera, using flow cytometry to measure Ki67 expression and assess the proliferation characteristics of stem cells (34). The authors suggest that not only do hematopoietic stem cells undergo hyperproliferation, but there is also a lower grade of apoptosis, thus characteristic of myeloproliferative neoplasms.

CONCLUSION

It is concluded that metformin, as a pleiotropic drug with potential anti-inflammatory and anticancer functions, is potentially essential to be used in association or even in the initial treatment of low-risk ET populations, considering that the drug may have long-term antithrombotic effects and decrease the morbidity of patients with the disease.

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ILLUSTRATIONS

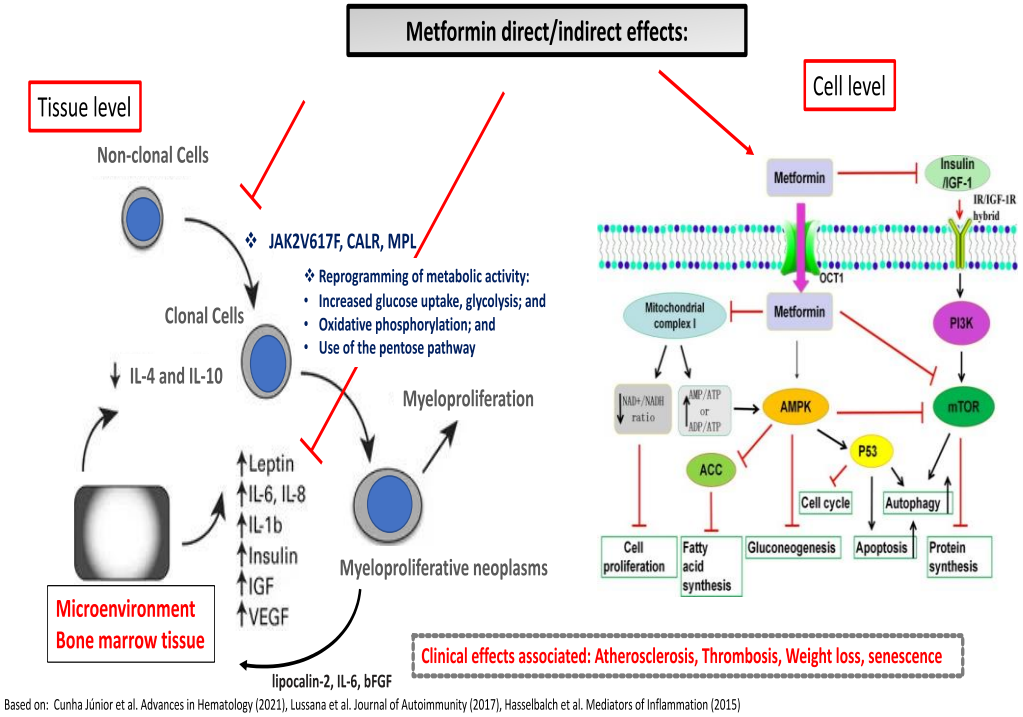

Figure 1. The Rationale Effect of Metformin on Essential Thrombocythemia

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