Gene expression analysis of autophagy genes in polycythemia vera patients

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GENE EXPRESSION ANALYSIS OF AUTOPHAGY GENES IN POLYCYTHEMIA VERA PATIENTS

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Assoc. Prof. Ivana Novak Nakir, PhD

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1 INTRODUCTION

1.1 Myeloproliferative neoplasms

Myeloproliferative neoplasms are a group of the malignant blood diseases. These neoplasms show similarities both in their clinical manifestations, as well as in their genetic drivers. They are characterized by a change in the genetic information, which leads to disturbances in the production of specific cell types in the blood. These changes, or mutations, cause myeloid hematopoietic stem cells to proliferate without restriction (1). The main mutations in myeloproliferative neoplasms (MPNs) are found in the JAK2 gene (Janus kinase 2), CALR gene (Calreticulin) or MPL gene (Thrombopoietin receptor). There are several types of myeloproliferative neoplasms being recognized: polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (MF). In respect of the mutations, most polycythemia vera patients are positive for the mutation of JAK2, which results in JAK2V617F. JAK2 is associated with the receptors for key hematopoietic cytokines, such as erythropoietin, thrombopoietin, and granulocyte colony-stimulating-factor. The mutation leads to the lack of inhibition of these receptors and therefore, a constant activation and proliferation of myeloproliferative cells (23).

1.1.1 Types

As already mentioned, several diseases are considered myeloproliferative neoplasms: polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (MF). Even though, they share many features, there are characteristics that are specific for the particular type.

1.1.2 Polycythemia vera

“Polycythemia vera” is a literal translation from Greek and means “too many cells in the blood” and refers to an increase in the red blood cell (RBC) mass, caused by a mutation in the JAK2 gene. It is frequently used interchangeably with the term erythrocytosis (1). PV is characterized by an insidious onset, an increased incidence in the elderly, erythrocytosis and sometimes leukocytosis, thrombocytosis and splenomegaly. In contrast to other hematologic malignancies, PV patients have a prolonged survival, if RBC and platelet counts can be controlled. Most patients are diagnosed accidently after a routine blood work was performed. They are usually diagnosed asymptomatic and become symptomatic if left untreated. Classic symptoms are related to the excessive production of RBCs and include arterial and/or venous thromboses, aquagenic pruritus and symptoms related to splenomegaly. After years, the erythrocytotic phase of the disease often inactivates and the excessive production of RBCs

ceases. Thereafter, patients can develop post-PV MF and/or acute leukemia. In the case of MF, the bone marrow is replaced with scar- and connective tissue and loses its ability to produce red blood cells (5).

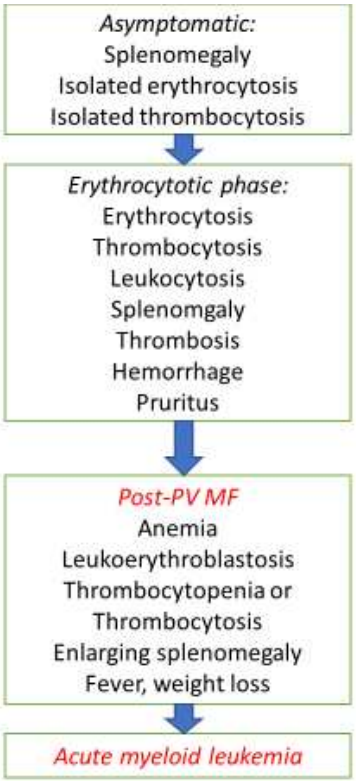


Figure 1. The progression of polycythemia vera. MF, myelofibrosis; PV, polycythemia vera.

In the case of MF, the bone marrow is replaced with scar- and connective tissue and loses its ability to produce blood cells (5).

1.1.2.1 Epidemiology

PV has an incidence of three cases per 100.000 in adults but is rarely seen in children, with fewer than 0.1% of PV patients diagnosed before 20 years of age (2). It is most commonly found in elderly patients, who are older than 60 years of age (3). Patients diagnosed with PV have a relatively long median survival of about fourteen years (4). Only five percent of patients with PV are younger than 40 years of age (5).

It occurs more often in men than it does in women. In rare cases PV runs in families and may be inherited in an autosomal dominant manner (6). Moreover, the prevalence was found to be higher among American Jews and lower among African Americans and Hispanics. The incidence is greater among Ashkenazi Jews, who originate from Europe, than among Arabs

and Sephardic Jews. Interestingly, extremely low occurrence rates have been reported from Japan, except in populations who were exposed to atomic bomb explosions. Additionally, higher rates were observed in chemical plant workers, leading to the conclusion that environmental factors like radiation- and toxin exposure do play a role (5).

1.1.2.2 Etiology

A mutation in the JAK2 gene, is the main reason for PV. The JAK-STAT pathway, which is affected by this mutation, is the principle signaling mechanism for a wide array of cytokines and growth factors. JAK activation stimulates cell proliferation and differentiation. Mutations like the one found in PV, will constitutively activate JAK and cause the myeloid hematopoietic stem cell to constantly proliferate (2,7).

1.1.2.3 Clinical manifestations

The major clinical manifestations of PV can be attributed to the overproduction of blood cells belonging to each of the myeloid progenitor cells which are affected by the malignant process. Typical symptoms for malignant diseases like night sweats, anorexia and fatigue may develop as well. RBCs are primarily affected, but thrombocytosis and lymphocytosis may occur. The symptoms are very diverse, as the blood interacts with virtually all organs in our body. Patients may present with symptoms including headaches, weakness, pruritus, dizziness, excessive sweating, visual disturbances, paresthesia, joint symptoms like gout, abdominal distress, weight loss and a thrombotic or hemorrhagic episode. Weight loss and fatigue, due to the consuming malignant disease, are common. Arthropathies and gout, due to the increased breakdown of blood cells and therefore also the release of uric acid into the blood, are also frequently seen. Even though there is an overproduction of RBCs, ruddy cyanosis can occur. This happens, because there are RBCs, but many of them are malformed and not mature. BCS is another complication of PV. Thrombotic events are common, even in usual sites like the splanchnic vessels. Occlusion of the portal veins may lead to BCS and hepatic cirrhosis. Splenomegaly is seen since lots of RBCs in the blood are not in the normal shape and therefore, they get caught by the spleen. Because of the massive amount of blood cells, there is a pro-thrombotic environment and simply a more viscous blood that often causes neurological symptoms derived from either necrotic or thrombotic events in the central nervous system (CNS). Some of those are transient ischemic attacks, cerebral infarction, fluctuating dementia, chorea, confusional states, visual disturbances, headaches and dizziness. Another symptom is erythromelalgia, which is an intermittent blocking of peripheral vessels in the extremities which leads to

recurrent pain. This is most probably caused by the malfunction of the platelets. Phlebotomy alone does not help, instead antiplatelet therapy and reducing the platelet counts is the treatment of choice. Patients with PV are at great risk of having complications during pregnancy. Thrombosis, maternal and fetal complications have been reported. PV patients might find it intolerable to bath or to come in contact with water in other occasions, because it may induce attacks of intense pruritus. Therefore, some patients tend to substitute bathing with gentle skin swabbing or simply not taking a bath. The mechanism responsible for the pruritus is not certain, although one reason for this might be the iron deficiency resulting from RBC production and destruction. Therefore, iron supplementation often results in symptomatic improvement. With time, PV causes post-PV myelofibrosis. Due to the proliferation and dying of the hematopoietic progenitor cells in the bone marrow, bone marrow fibrosis occurs and causes anemia. This anemia is mostly caused by splenic pooling, ineffective erythropoiesis, hemolysis and extramedullary production of RBCs with a shortened life span. Considering the laboratory manifestations, most PV patients have elevated counts of white blood cells (WBCs), RBCs and platelets. If hematocrit values go beyond 48% in females and 49% in males, further investigation should be started. Sometimes though, the diagnosis can be more difficult, since iron deficiency can lead to a normal hematocrit in PV patients. Also, the hematocrit might be normal despite an increased number of RBCs. This is due to portal hypertension, which causes the plasma volume to expand and dilute the RBCs. If bone marrow biopsies are taken from PV patients, marked hypercellularity, erythroid and megakaryocytic hyperplasia with pleiomorphic enlarged megakaryocytic will most likely be found and hence are the hallmarks of MPN (Figure 1) (8).

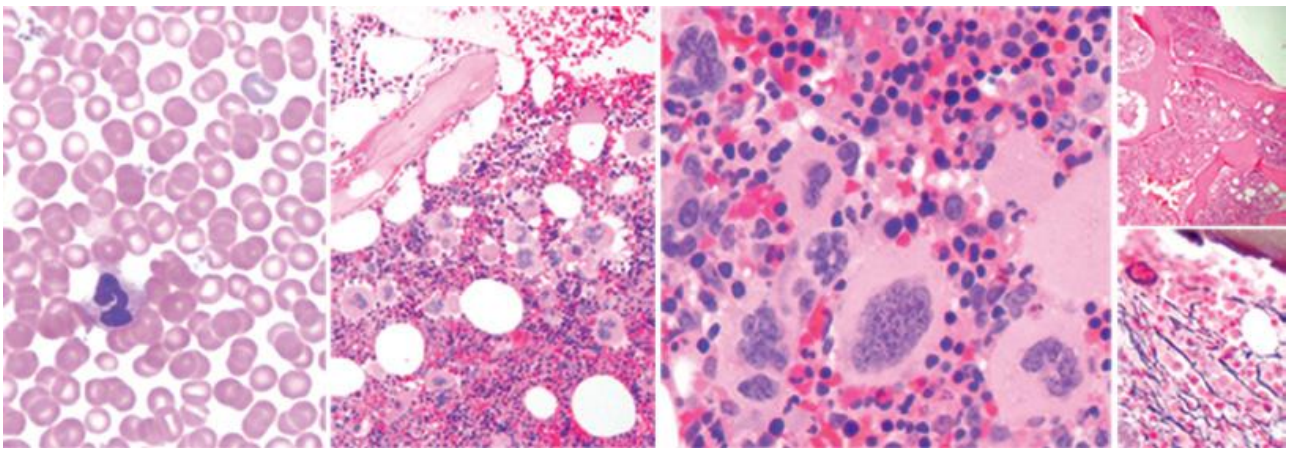


Figure 2. Photomicrograph of bone marrow biopsy obtained from a patient with polycythemia vera in myelofibrotic phase demonstrating hypercellularity and increased number of megakaryocytes (x160)

(Hoffmann R, Benz E, Silberstein L, Heslop H, Weitz J, Anastasi J et. al. Other myeloproliferative neoplasms. In: Hematology: Basic Principles and Practices. 7th Edition. 2017. p.1094).

1.1.2.4 Treatment

Even with modern treatment, the mortality rate of PV patients is increased 1.84 times compared to the age- and sex-matched population. PV patients have a cumulative survival of 15 to 17 years after diagnosis. For now, the most important goals in therapy are to reduce the risk of thrombosis, reduce the severity of PV symptoms (e.g. pruritus), and to prevent the progression into MF. Depending on the patients risk of developing another thrombotic event, different therapy plans can be followed. First of all, testing should confirm the PV diagnosis, in order to avoid inappropriate exposure of patients with nonmalignant disorders to myelosuppressive treatment. If confirmed, low-risk patient receive phlebotomy with an anti-platelet medication, whereas high-risk patients receive both of those and a myelosuppressive therapy additionally (e.g. hydroxyurea, IFN, busulfan, melphalan, ruxolitinib) (9). The current therapeutic goals are to reduce the risk for thrombosis by reducing the hematocrit down to 45% in men and to 42% in women (10). To reduce the hematocrit as soon as possible, 250-500 ml of blood can be withdrawn every other day. Elderly patients with cardiovascular or pulmonary comorbidities should be phlebotomized more carefully (twice a week). Hyperuricemia, resulting from the breakdown of RBCs can be treated with allopurinol (100-300 mg/day). In patients with severe pruritus, ruxolitinib can be used additionally to the common treatments of PV. If any surgery or dental procedure is planned, it should be postponed until the hematocrit has normalized for at least two months. One week before the surgery, the aspirin medication should be withdrawn. If there is a need for an emergency surgery, phlebotomy and cytapheresis (the removal of blood cells via apheresis devices) should be initiated. To avoid a teratogenic effect in men and women who are planning to have children, only phlebotomy, aspirin and IFN-alpha should be used in the treatment regimen. Ruxolitinib, a small-molecular inhibitor of JAK1/2 was approved in the US in 2015 for PV patients who cannot tolerate hydroxyurea. In PV patients who developed BCS, portal vein decompression is the only treatment currently available and effective. Of course, prevention with maintenance of normal blood values is the optimal approach. As a stem cell disorder, PV can also be treated with a stem cell transplantation. Usually patients are transplanted once PV has developed into MF, MDS or acute leukemia (9).

1.1.2.5 Prognosis

A prognosis is always based on the individual patient and the course of PV in him/her. Depending on how well PV is treated, the survival time varies greatly. In a study from the 1970s, the median survival time from the onset of symptoms was only 1.5 years in untreated patients. This value is probably not valid, because many patients are diagnosed in routine check-ups and have been asymptomatic for long periods. When treated appropriately, PV is associated with a survival period of about 17 years. Increasing age, a history of thrombosis and cardiovascular risk factors are the main determinants of the risk stratification for additional thromboses in PV patients (Figure 3).

Risk Category	Age > 60 Years or History of Thrombosis	Cardiovascular Risk factors*
Low	No	No
Intermediate	No	Yes
High	Yes	

Figure 3. Risk stratification in PV based on thrombotic risk. The older the patient is and the more cardiovascular risk factors are present, the higher is the risk for additional thromboses in PV patients.*Hypertension, hypercholesterolemia, diabetes, and smoking. Extreme thrombocytosis (platelet count >1500 x 10^9 L^-1) is a risk factor for bleeding. Its role as a risk factor for thrombosis is uncertain. An increasing leukocyte count has been identified as a novel risk factor for thrombosis, but confirmation is required. Data from Finazzi G, Barbui T: How I treat patients with polycythemia vera. Blood. 2007;109: p.5104

1.1.3 Other types

Chronic myeloid leukemia, essential thrombocythemia and myelofibrosis also belong to the myeloproliferative neoplasms. They shall be addressed shortly considering their clinical and pathogenetical entities. Especially myelofibrosis is important to mention because of its strong connection to both PV, essential thrombocythemia and chronic myeloid leukemia.

1.1.3.1 Essential thrombocythemia

ET is a myeloproliferative neoplasm characterized by excessive platelet counts, bone marrow megakaryocyte hyperplasia, leukocytosis, splenomegaly, thrombotic and hemorrhagic events and possible progression to myelofibrosis.

1.1.3.1.2 Epidemiology:

The incidence is estimated to be around 1.5-2.4 patients per 100.000 populations annually. Like PV, ET is a neoplasm of the elderly. The median age at diagnosis is 67 to 73 years.

1.1.3.1.3 Etiology:

Like in PV, a mutation affecting the JAK-STAT signaling pathway leads to hematopoiesis in absence of exogenous cytokines. The JAK2V617F mutation occurs in 50-60% of patients, whereas other mutations, like the mutation of the MPL gene, making up another 30% of patients, leaving only about 10% of patients without one of those mutations. The MPL gene is responsible for the cell surface receptors on megakaryocytes and other progenitor cells. If the receptor is mutated, it can trigger the cell to produce thrombocytes without an external trigger, as well.

1.1.3.1.4 Clinical manifestations:

The symptoms are quite variable. Many patients are first diagnosed by accident, at their regular check-up. Small- or large-vessel thrombosis, as well as neurological complications like headaches, paresthesia, cerebral circulatory ischemia and more are seen commonly. The laboratory hallmark for ET is the sustained and unexplained elevation of the platelet count (>450 x 10^9).

1.1.3.1.5 Treatment:

As in PV, the goal is to prevent additional thrombotic events and to decrease the risk of progression to myelofibrosis, myelodysplastic syndrome or acute myelocytic leukemia (AML). In the literature, there is a big controversy about who to treat and what platelet numbers should be aimed for. Especially for the asymptomatic patients, it remains largely problematic. Patients with thrombotic or hemorrhagic events is, in contrast, very clear. Those patients need platelet pheresis and a myelosuppressive treatment. Smoking cessation plays a crucial part as well, as there is a large number of thrombotic events seen in smokers. Usually hydroxyurea is started at a dose of 2-4 g/day, together with the platelet pheresis.

1.1.3.1.6 Prognosis:

The 10-year survival of ET patients ranges from 64% to 80%. Compared to PV, the prognosis is not as bad and the main risk factors for adverse outcomes are similar, which here are advanced age and previous thrombotic events. A major risk is the progression to MF or AML (11).

1.1.3.2 Myelofibrosis

Primary myelofibrosis is a part of the myeloproliferative neoplasms. Therefore, it's a malignant, stem cell-derived clonal myeloproliferation that is often accompanied by JAK2, CALR or MPL mutation. Moreover, bone marrow fibrosis, anemia, hepatosplenomegaly, extramedullary hematopoiesis, constitutional symptoms, cachexia, leukemic progression and shortened survival are characteristics of MF (12).

1.1.3.2.1 Epidemiology:

The incidence of MF in Europe, North America and Australia ranges from 0.5 to 1.3 cases per 100.000 persons. Significant higher numbers of cases in the proximity of Hiroshima and in patients who received the contrast material Thorotrast, which contains radioactive material that is taken up and stored indefinitely in the reticuloendothelial system, indicated a strong link between excessive radiation exposure and the development of MF. The average age at diagnosis is between 50 and 69 years of age. So just like PV and ET, MF is predominantly a neoplasm of the elderly.

1.1.3.2.2 Etiology:

The mutations typical for the other myeloproliferative neoplasms are also of big importance in this neoplasm. JAK2V617F, MPL and CALR are genes which can be mutated and causative for MF. Both PV and ET can eventually lead to MF.

1.1.3.2.3 Clinical manifestations:

About a quarter of all patients are completely asymptomatic and are only being diagnosed because of an enlarged spleen, an abnormal blood cell count or peripheral blood smear. Typical symptoms are fatigue, nights sweats, weight loss, symptoms due to an enlarged spleen, bleeding, gout or renal stones, pallor, spleno- and hepatomegaly and petechiae. The enlarged spleen can cause delayed gastric emptying and decreased appetite. The pressure on the colon can lead to severe diarrhea.

1.1.3.2.4 Treatment:

Treatment is reserved for the patients with intermediate to high risk disease. Patients who should be treated include patients with symptoms like anemia, bleeding problems, significant hyperuricemia, bone pain, systemic symptoms, portal hypertension and life-threatening gastric bleeding. Gout and hyperuricemia should be treated with hydration and chronic administration of allopurinol (300mg/day). If given early in the course, IFN-alpha might be able to slow down the progression of the disease by decreasing the fibrosis in the bone. A therapy with corticosteroids was used successfully in patients with hemolytic anemia associated with PMF. Ruxolitinib may be used, especially for patients with splenomegaly related symptoms.

1.1.3.2.5 Prognosis:

The median overall survival after the diagnosis of MF is approximately 6-7 years. Infection, leukemic transformation, heart failure, bleeding, hepatic failure, portal hypertension, renal failure and pulmonary embolism are the primary causes of death (13).

1.1.3.3 Chronic myeloid leukemia

CML is a myeloproliferative neoplasm that, when untreated can lead to a depletion of hematopoietic stem cells, especially white blood cells and platelets. CML is consistently associated with the Philadelphia chromosome, a cytogenetic abnormality which leads to the BCR-ABL fusion oncogene.

1.1.3.1.1 Epidemiology/Etiology:

The Philadelphia chromosome (PH) is a translocation from chromosome nine to chromosome 22 [t (9;22)]. Having this chromosomal translocation, an oncogene called BCR-ABL is formed. It was found in the 1960s and was named after the city of its' discovery. All CML patients have this mutation. CML is the most common myeloproliferative neoplasm and has an incidence of 1.5 cases per 100.000 population per year. The median age at diagnosis is 67 years and the incidence increases with age. The mechanism of Ph first formation and time needed for it to cause overt disease is unknown.

1.1.3.1.2 Clinical manifestations:

At diagnosis, most patients are in the chronic phase. They usually show fatigue, weight loss, bone pain, sweating, abdominal discomfort and early satiety related to splenomegaly. At this time, the blood count will show leukocytosis as well as thrombocytosis

and anemia. Having two further phases (accelerated phase; blast crisis), it will show a variety of symptoms. In the accelerated phase, fever, night sweats, bone pain, difficulties controlling the blood count with conventional therapy, increased numbers of blasts and early myeloid cells in marrow and peripheral blood are usually seen. The blast crisis resembles acute leukemia. It is defined as having more than 20% blasts in the bone marrow or peripheral blood and the presence of aggregates of blasts in the bone marrow biopsy.

1.1.3.1.3 Treatment:

Several treatment options are available. In short, following broad treatments are used: chemotherapy, interferon and tyrosine kinase inhibitors. A complete hematologic response to treatment is defined as the achievement of normal WBC and platelet counts, a normal differential blood count and cessation of all symptoms related to CML.

1.1.3.1.4 Prognosis:

Prognostic tests have been developed to predict the length of the chronic phase in the patients. The algorithm usually includes the size of the spleen, percentage of circulating blasts, platelet count and age (19). Generally, around 75% of CML patients survive for five years or longer after being diagnosed (20).

1.2 JAK2 mutation in Polycythemia vera

A mutation in the JAK2 tyrosine kinase is the main mechanism behind PV and the other myeloproliferative neoplasms. A tyrosine kinase is an enzyme that mediates the transfer of phosphate from ATP to the amino acid tyrosine on a protein, i.e. STAT (Signal transducers and activators of transcription), to activate it and produce cell signaling transduction. It can function as an “on”/“off” switch (14). The first discovery of the significance of the JAK2 mutation in PV was found by Vainchenker and colleagues in France (15). They observed that the inhibition of JAK2 reduced the EPO-independent colony formation by PV bone marrow mononuclear cells. After this discovery, they sequenced the hematopoietic cells of PV patients and found a single recurrent point mutation. This was a guanine-to-thymine which resulted in a substitution of valine to phenylalanine at codon 617 in the pseudokinase domain (JH2) of JAK2, which resulted in the JAK2V617F signaling in myeloproliferative neoplasms (Figure 3,4) (16). The specific JAK2V617F mutation can be detected in about 90% of PV patients and therefore, it is a significant marker as stated before (8).

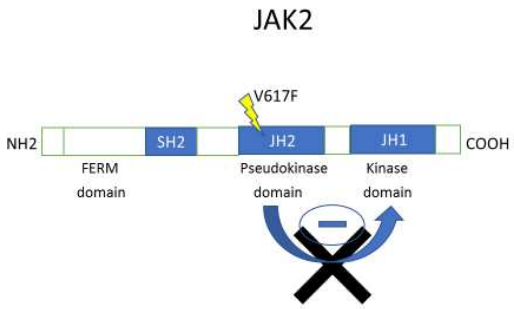


Figure 4. Structure of *JAK2V617F*: the mutation is located in pseudokinase JAK. JH2 and disrupts the autoinhibition of this regulatory domain. Consequently, the tyrosine kinase corresponding to the JH1 domain is constitutively activated.

PV is probably not solely initiated by the JAK2V617F mutation. Since this mutation is present in the other MPNs as well, it is suggested that other genetic or epigenetic events likely contribute to the initiation of PV. This suspicion was raised when researchers found a cluster of JAK2V617F-positive PV patients in a specific region in Pennsylvania where numerous hazardous materials, i.e. waste coal power plants and United States Environmental Protection Agency superfund sites, were present. Moreover, a higher incidence of PV was seen in Japanese populations which were exposed to atomic bomb explosions, indicating that radiation exposure is a contributing factor for PV (17).

1.3 Autophagy and mitophagy in myeloproliferative diseases

Autophagy is a cellular pathway for the sequestration of old organelles and protein aggregates from the cytoplasm and their delivery into degradation lysosomes. It plays an important role in several mechanisms like starvation, immunity and the development of cancer. Studies performed on mice revealed that autophagic defects in hematopoietic stem cells (HSCs) may cause leukemia. Also, mice lacking the autophagy gene Atg7 developed a myeloproliferative syndrome which resembled the human myelodysplastic syndrome (MDS), which progressed to acute myeloid leukemia. The selective removal of mitochondria by autophagy is termed mitophagy and was confirmed to be essential for erythroid development. Myelodysplastic syndrome, which is another myeloproliferative neoplasm, is characterized by mitochondrial abnormalities, implying possible defects in mitophagy. Studies suggest that nonfunctional autophagy and mitophagy leads to an accumulation of damaged mitochondria

and reactive oxygen species which itself leads to the cell death of many progenitor cells and possibly transformation of some surviving ones (24).

Different autophagy/mitophagy genes have been identified. They are either specific for autophagy or mitophagy, respectively. In the group of autophagy genes, both specific autophagy genes and general ones have been found.

The general autophagy markers are the following: LC3A, LC3B, Atg5 and BECN1. The LC3 genes convert to microtubule associated proteins which mediate the physical interaction between microtubules and components of the cytoskeleton (26). Atg5 encodes a protein that is crucial in autophagic vesicle formation (27). BECN1 encodes a protein that is part of the phosphatidylinositol-3-kinase complex, which mediates vesicle-trafficking processes (28). The selective autophagy marker is SQSTM1/p62, which can recognize the specific, targeted cargo within phagolysosomes (25). It is implicated in the activation of the transcription factor NF-κB. The modulation of p62 seems to be a key factor in tumorigenesis (22). Mitophagy specific genes are Nix and Bnip3. With the Nix gene, a protein is produced that targets mitochondria and causes apoptotic changes, including loss of membrane potential and the release of cytochrome c (29). Bnip3 encodes a protein that acts as a pro-apoptotic factor and interacts with anti-apoptotic proteins to silence them. This gene is silenced in tumors by DNA methylation (30.) Both have a critical role in the pathogenesis of cancer and heart disease. Both are known to be able to also induce autophagy, where they play a role as autophagy receptors specifically targeting mitochondria for degradation (31). In erythroid cells, Nix is needed for mitophagy. Whereas in hypoxia, it is both NIX and BNIP3 which induce mitophagy (21,32).

2 OBJECTIVE

The aim of the study was to analyze the expression of autophagy related genes in polycythemia vera patients. This was done under the premise that the processes of autophagy are affected in polycythemia vera patients. The study tested this hypothesis by measuring the expression of representative autophagy and mitophagy marker genes (Atg5, BECN1, Nix, Bnip3, LC3A, LC3B and SQSTM1/p62) in erythrocytes taken from blood samples of the participants.

3 MATERIALS AND METHODS

3.1 Ethical background of data collection

All data which were used for this thesis were gathered at the Department of Pathology and the Department of Immunology and Medical genetics, University of Split, School of Medicine. The collection of samples and data was in accordance with the ethical standards of the institutional research committees (University Hospital Split and School of Medicine in Split) and with the 1964 Helsinki declaration.

3.2 Patients

The study enrolled eight patients, four of whom were diagnosed PV patients and the remaining four had leukemia. The PV patients were JAK2 positive and the leukemia controls were JAK2 negative, but Philadelphia chromosome positive. Blood was withdrawn from all patients and sent to the Department of Pathology, University of Split, School of Medicine. PV and leukemia were diagnosed, and the samples were sent to the Department of Immunology and Medical genetics, University of Split, where all further laboratory work was done.

3.3 Materials and methods

The patients' blood samples were obtained by the Department of Pathology, University of Split, School of Medicine, where total RNA was isolated and real time quantitative PCR (RT-PCR) performed to generate cDNA that was sent to the Department of Immunology and Medical Genetics, University of Split. Real time PCR was used to analyze the gene expression in a sample. To perform the RT-PCR, a microplate, together with pipettes (Eppendorf, Hamburg, Germany) were used to mix together the cDNA probe, the SYBR Green master mix, the primer for each gene of interest and distilled water. Afterwards, the microplates were centrifuged. The microplate was then inserted into the qPCR machine (Applied biosystems; Thermo Fisher Scientific, Waltham, USA). After running through the cycles of PCR and checking the amount of fluorescent dye being detected after each cycle, the Ct (Cycle threshold) was obtained. If, i.e., the fluorescent light was detected after 20 PCR cycles, the cycle threshold is 20. The smaller the Ct, the earlier the fluorescent light is detected and the higher the expression of that particular gene.

The Ct of each gene, together with the values for the housekeeping gene RPS23 were used to calculate 2^-deltaCt in Excel (Microsoft Corp., Redmont, WA, USA). Housekeeping genes should constantly have the same expression pattern, which is then used for the analysis. RPS23, specifically, is a gene that encodes a ribosomal protein. After attaining 2^deltaCt for each gene, a multiple comparison graph was created, which displayes the 2^-deltaCt values of the gene (e.g. Nix) in JAK2 negative and JAK2 positive patients (IBM SPSS, Armonk, New

York, USA) is generated. This way, the expression of particular genes was compared among the two groups of patients.

3.4 Statistical analysis

Statistical analysis was performed using the statistical software SPSS (IBM, Armonk, New York, USA). Continuous data were presented as median, whereas categorial variables were presented as whole numbers and percentages. The statistical significance was defined as $P<0.05$.

4 RESULTS

The study included eight patients, four having PV and four suffering from leukemia. The PV patients were JAK2 mutation positive, whereas the leukemia patients were negative. Seven genes/markers of autophagy/mitophagy were analyzed in these patients. Overall, four out of seven genes showed a higher 2^-deltaCt value in PV patients, therefore they were expressed less in these patients. By analyzing the Ct values of individual genes (Atg5, BECN1, LC3A, LC3B, SQSTM/p62, Nix and Bnip3) and comparing them with the Ct value of genes for expression standardization (RPS23), relative values of changes in expression of target genes in PV patients (JAK2 positive) and leukemia patients (JAK2 negative) were obtained.

4.1 General autophagy markers

4.1.1 Atg5 in JAK2+/JAK2-

No statistically significant difference was found in the expression of Atg5 in the blood ofJAK2-positive and JAK2-negative patients.

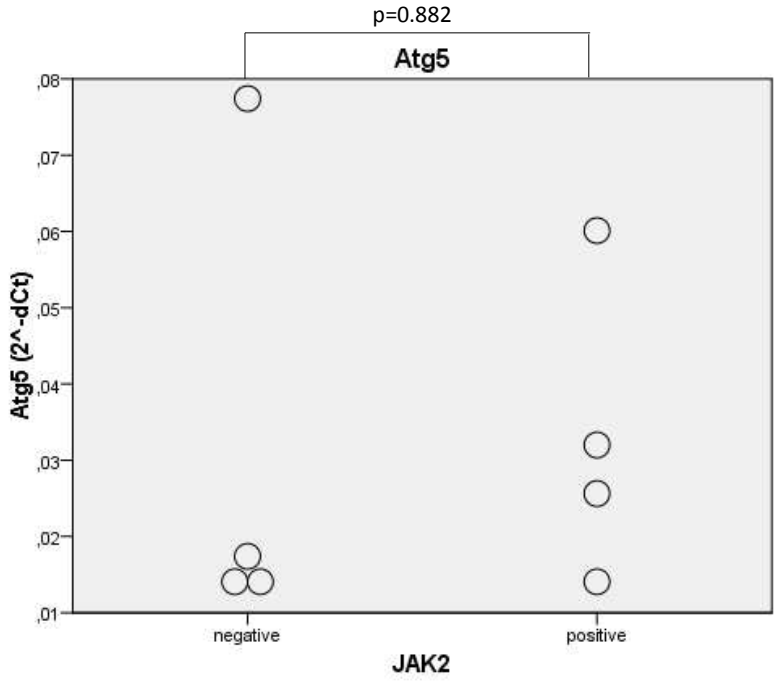


Figure 5. Gene expression of Atg5 in blood of JAK2 positive and JAK2 negative patients. There is no statistically significant difference (P=0.882) between the two groups.

4.1.2 BECN1 in JAK2+/JAK2-

No statistically significant difference was found in the expression of BECN1 in the blood of JAK2-positive and JAK2-negative patients.

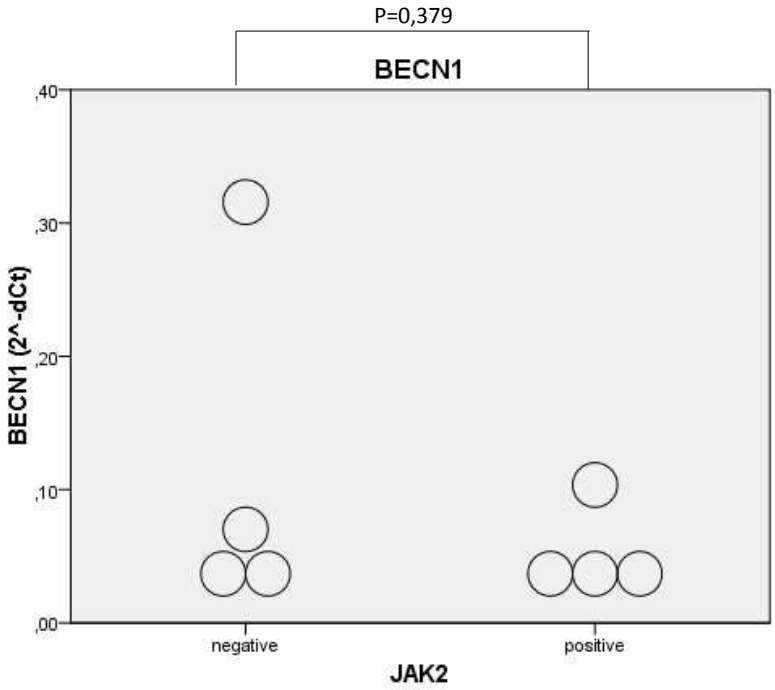


Figure 6. Gene expression of BECN1 in blood of JAK2 positive and JAK2 negative patients. There is no statistically significant difference (p=0.379) between the two groups.

4.1.3 LC3A in JAK2+/JAK2-

No statistically significant difference was found in the expression of LC3A in the blood of JAK2-positive and JAK2-negative patients.

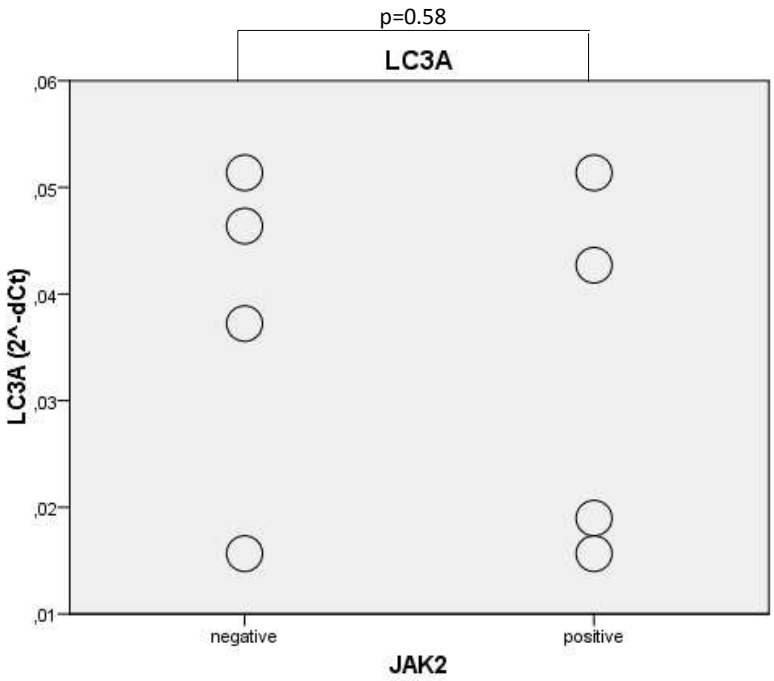


Figure 7. Gene expression of LC3A in blood of JAK2 positive and JAK2 negative patients. There is no statistically significant difference (P=0.58) between the two groups.

4.1.4 LC3B in JAK2+/JAK2-

No statistically significant difference was found in the expression of LC3B in the blood of JAK2-positive and JAK2-negative patients.

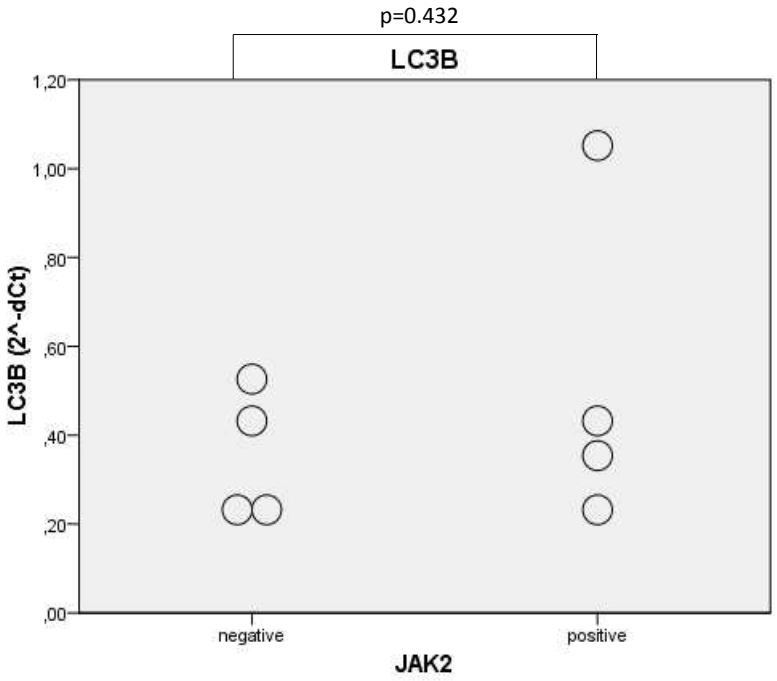


Figure 8. Gene expression of LC3B in blood of JAK2 positive and JAK2 negative patients. There is no statistically significant difference (P=0.432) between the two groups.

4.2 Selective autophagy markers

4.2.1 SQSTM1/p62 in JAK2+/JAK2-

No statistically significant difference was found in the expression of SQSTM1/p62 in the blood of JAK2-positive and JAK2-negative patients.

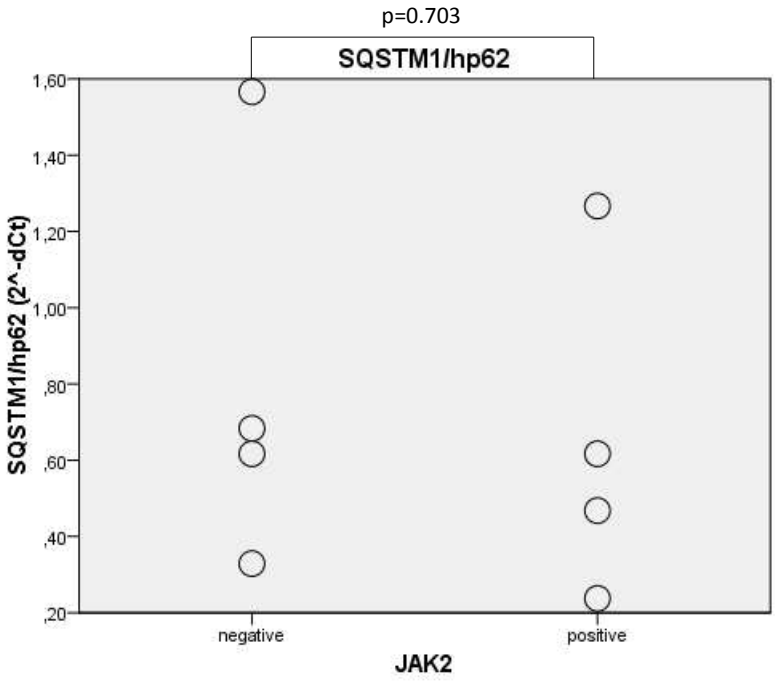


Figure 9. Gene expression of SQSTM1/p62 in blood of JAK2 positive and JAK2 negative patients. There is no statistically significant difference (P=0.703) between the two groups.

4.3 Receptor-mediated autophagy markers

4.3.1 Nix in JAK2+/JAK2-

No statistically significant difference was found in the expression of Nix in the blood of JAK2-positive and JAK2-negative patients.

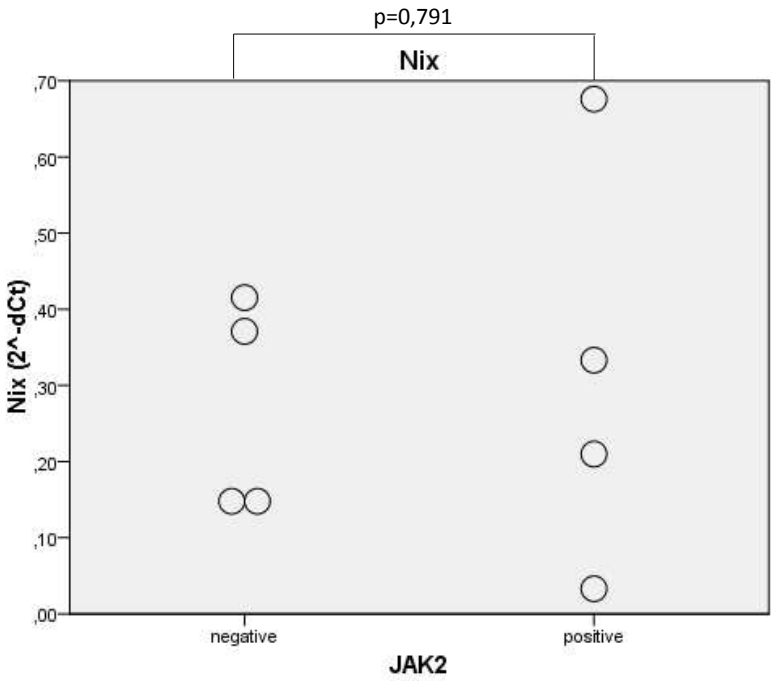


Figure 10. Gene expression of Nix in blood of JAK2 positive and JAK2 negative patients. There is no statistically significant difference (P=0.791) between the two groups.

4.3.2 Bnip3 in JAK2+/JAK2-

No statistically significant difference was found in the expression of Bnip3 in the blood of JAK2-positive and JAK2-negative patients.

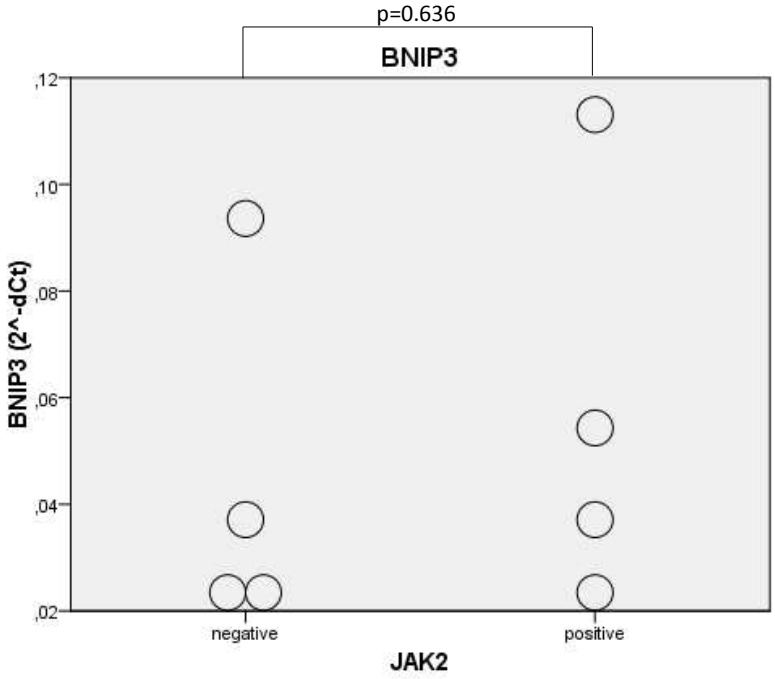


Figure 11. Gene expression of BNIP3 in blood of JAK2 positive and JAK2 negative patients. There is no statistically significant difference (P=0.636) between the two groups.

5 DISCUSSION

This study included eight patients who were either diagnosed with PV or leukemia, respectively. Four of them were JAK2 mutation positive (PV) and the remaining four were JAK2 mutation negative (Leukemia controls). We found a tendency for decreased expression of four autophagy related genes, ATG5, LC3B, NIX and BNIP3 in JAK2 positive patients, compared to the JAK2 negative group. Two (LC3B, Atg5) out of four general autophagy genes were expressed less in JAK2 positive patients than in JAK2 negative ones. The selective autophagy gene SQSTM1/p62 showed no difference in expression among the two groups of patients. Whereas the mitophagy genes showed decreased expression in JAK2 positive PV patients, compared to the leukemia control. On the other hand, both BECN1 and LC3A showed an increased expression in PV patients compared to leukemia controls. Since this study only included eight patients, evaluating the measured statistical significance and therefore the value of our results is critical. The small sample size might be the limiting factor in generating significant results. In order to confirm the outcome, further research with appropriate sample sizes are due. This study investigated the expression of autophagy genes in all blood cells; therefore, the study should be repeated with erythrocyte-enriched samples, to give a better perspective of gene expression in erythrocyte population only. In addition, the bone marrow from the same patients could be analyzed to reach the statistically significant results and clarify the autophagy-related genes expression pattern. If, however, these results were to be found significant, this would implicate a decreased autophagy/mitophagy in polycythemia vera patients, leading to concomitant formation of immature red blood cells. Previously conducted research has indeed pointed in the same direction, indicating that autophagy might be altered in myelodysplastic syndromes, including polycythemia vera. Researchers from China performed research on hematopoietic cells of twelve newly diagnosed PV patients with JAK2 V617F mutation and found a high basical activity of autophagy (33). Another team, also from China, undertook retrospective analysis of electron microscopy specimens of 3277 patients with hematological diseases and found that nucleated erythrocyte autophagy occurred more frequently among patients with myelodysplastic syndromes (34). The disturbed autophagy process seems to be one essential characteristic in the pathogenesis of polycythemia vera. Future studies might be able to pinpoint the particular role and importance of either increased or decreased autophagy gene expression and explore ways to induce, or, respectively, reduce its activity in order to modify the course of the disease.

6 CONCLUSION

In this study, we couldn't find a significant difference in autophagy gene (Atg5, LC3A, LC3B, Nix, Bnip3, BECN1, SQSTM1/p62) expression between PV and leukemia patients. Nevertheless, we have observed, that specific genes are more, and some are less frequently expressed in polycythemia vera patients, compared to the leukemia control. Further studies are needed to evaluate the significance of the outcome and if there is a correlation between autophagy gene expression and polycythemia vera.

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8 SUMMARY

Objectives: There is an increasing interest in the correlation between autophagy/mitophagy processes in myeloproliferative neoplasms like polycythemia vera. Especially the Department of Immunology and Medical Genetics at the University of Split, School of Medicine conducts intensive research in this field. The aim of the study was to analyze the expression of autophagy/mitophagy related genes in PV patients in order to find out, if a difference is observable compared to a control group.

Materials and methods: We used blood samples from eight PV patients to test the expression of seven autophagy/mitophagy related genes via qPCR. We then differentiated into JAK2 positive and negative patients and analyzed accordingly.

Results: After performing qPCR and data analysis, we could see that some genes in JAK2 positive PV patients were expressed more and some less than in the leukemia control. The results were not statistically significant.

Conclusion: The decreased expression of some autophagy and mitophagy related genes in PV patients could be an indicator for the importance of auto- and mitophagy in PV. Especially Nix and Bnip3, which both showed a decreased expression, might be indicative, because they already have been shown to play a crucial role in cancer and heart disease. Further studies are needed to evaluate the significance of this relationship.

9 CROATIAN SUMMARY

Cilj istraživanja: Postoji povećan interes u povezanosti autofagije/mitofagije u mijeloproliferativnim neoplazmama poput policitemije vere. Posebice na Odjelu za imunologiju i medicinsku genetiku na Medicinskom fakultetu, Sveučilišta u Splitu provode opsežna istraživanja u tom području. Cilj ovog istraživanja bio je analizirati pojavnost gena povezanih s autofagijom/mitofagijom u pacijenata oboljelih od PV kako bi otkrili postoji li zamjetna razlika prema kontrolnoj skupini.

Materijali i metode: Koristili smo uzorke krvi od 8 pacijenata oboljelih od PV kako bismo pomoću qPCR testirali pojavnost sedam gena povezanih s autofagijom/mitofagijom. Zatim smo pacijente podijelili u JAK2 pozitivne i negativne i sukladno tome napravili analizu.

Rezultati: Nakon provedbe qPCR i analize podataka, primjetili smo da su neki geni u JAK2 pozitivnih pacijenata s PV izraženiji, a neki manje izraženi, nego u kontrolnoj skupini s leukemijom. Dobiveni rezultati nisu bili statistički značajni.

Zaključak: Smanjena ekspresija nekih gena povezanih s autofagijom i mitofagijom u pacijenata s PV može biti pokazatelj važnosti autofagije i mitofagije u PV. Pogotovo Nix i Bnip3, koji su oba bili manje izraženi, jer se već otprije pokazalo kako imaju važnu ulogu u nastanku karcinoma i bolesti srca. Daljnje studije su potrebne za procjenu važnosti ove povezanosti.

10 CURRICULUM VITAE

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