Abstract: This work analyses the results of research regarding the predisposition of genetic hematological risks associated with secondary polycythaemia. The subjects of the study were selected based on shared laboratory markers and basic clinical symptoms. JAK2 (Janus Kinase 2) mutation negativity represented the common genetic marker of the subjects in the sample of interest. A negative JAK2 mutation hypothetically excluded the presence of an autonomous myeloproliferative disease at the time of detection. The parameters studied in this work focused mainly on thrombotic, immunological, metabolic, and cardiovascular risks. The final goal of the work was to discover the most significant key markers for the diagnosis of high-risk patients and to exclude the less important or only complementary markers, which often represent a superfluous economic burden for healthcare institutions. These research results are applicable as a clinical guideline for the effective diagnosis of selected parameters that demonstrated high sensitivity and specificity. According to the results obtained in the present research, groups with a high incidence of mutations were evaluated as being at higher risk for polycythemia vera disease. It was not possible to clearly determine which of the patients examined had a higher risk of developing the disease as different combinations of mutations could manifest different symptoms of the disease. In general, the entire study group was at risk for manifestations of polycythemia vera disease without a clear diagnosis. The group with less than 20% incidence appeared to be clinically insignificant for polycythemia vera testing and thus there is a potential for saving money in mutation testing. On the other hand, the JAK V617F (somatic mutation of JAK2) parameter from this group should be investigated as it is a clear exclusion or confirmation of polycythemia vera as the primary disease.

Keywords: JAK2; mutation; polycythemia vera; secondary polglobulism

1. Introduction

Erythrocytes are one of the most important cells in the body due to their ability to transfer blood gases. They are nucleusless, biconcave-shaped blood elements formed in the red bone marrow, carrying hemoglobin. The life span is around 120 days. The average number of red blood cells in men is 4.3–5.7 10¹²/L and in women it is 3.8–4.9 10¹²/L. Some men have a sustained increase in hemoglobin levels without a diagnosis of the chronic progressive clonal myeloproliferative disease polycythemia vera (PV). This disease is characterized by a JAK V617F mutation and a chronic increase in erythrocyte production as a primary
disease. This paper focuses on the evaluation of hematological blood tests in patients prone to venous thrombosis and atherosclerosis with higher erythrocyte mass values.

Deep vein thrombosis is very difficult to diagnose and sometimes presents without significant difficulties. Most often it manifests itself as an embolism, or blockage of the artery. It is a partial or complete blockage of a deep vein by a blood clot, most often in the lower limb, very often leading to a second form of thromboembolic disease. It is most often recognized by unilateral swelling of the calf or the whole leg.

Thromboembolism is a condition where a blood clot has broken loose into the bloodstream and is closing a blood vessel. It can occur in venous system (venous thromboembolism) or in arteries (arterial thromboembolism).

Deep vein thrombosis is very dangerous, and anticlotting treatment must be started as soon as possible. Information on how thromboembolism occurs was described as early as the 19th century by the German physician Rudolf Virchow, who identified three main factors for the development of thrombosis (Figure 1).

**Figure 1.** Virchow’s triad, which describes the three categories of factors in thrombosis.

Myeloproliferative diseases specifically are PV, essential thrombocythemia (ET) and primary myelofibrosis. All these diseases are associated with an overproduction of blood elements.

A large number of patients with myeloproliferative disorders have been found to carry a dominant JAK2 mutation. This gene causes the expansion of hematopoietic progenitors in these disorders [1]. Furthermore, JAK V617F has been found to be a somatic mutation of hematopoietic cells. The JAK V617F mutation is most commonly found in patients with PV. Tefferi et al. [2] discussed the World Health Organization (WHO) criteria for the diagnosis of PV, ET, and primary myelofibrosis. All these diseases were described in their research using the older criteria and then described using the new proposed criteria. Baxter et al. [3] discussed acquired JAK2 tyrosine kinase mutations in human myeloproliferative diseases. They noted that finding the Val617Phe (point mutation of Janus kinase 2) mutation may become a good identifier to distinguish myeloproliferative diseases from other similar ones. Levine et al. [4] reported how JAK2 tyrosine kinase mutations are activated in myeloproliferative diseases. As a result, we found that inhibition of JAK2V617F leads to a reduction in hematopoietic cell proliferation. This tyrosine kinase is a potential target for drug therapy. Neunteufl et al. [5] described endothelial dysfunction in patients with PV. This endothelial dysfunction should be considered as a pathology in arterial thrombosis in polycythemia vera. Furthermore, Bonetti et al. [6] described the production of nitric oxide and the protection of the blood vessels against platelet and leukocyte adhesion. The study demonstrated that polycythemia vera is associated with endothelial dysfunction and may cause other arterial diseases. The review also discussed endothelial dysfunction as a feature of atherosclerotic risk factor [7]. Its dysfunction leads to impaired endothelium-dependent vasodilation. Endothelial nitric oxide synthase in coronary artery disease was also described. Polymorphisms in exon 7 of the eNOS (Endothelial NOS) gene are being studied. Finazzi et al. [8] discussed the risk of thrombosis in patients with ET and PV according to the JAK2V617F mutation. Due to the JAK2V617F mutation, ET is divided into two types. Ihle and Gilliland [9] compared the risk of thrombosis in ET and PV according
to JAK2 mutation status. The regulation of JAK2 kinase activity was also summarized, followed by the activation of JAK2 kinase by chromosomal translocation and mutations in myeloproliferative diseases. JAK2V617F is not the only genetic contributor to the disease phenotype. Again, the work proposed by Lippert et al. [10] dissected the observed group into the three diseases already mentioned and studied homozygosity and heterozygosity for JAK2 mutations. A brief work reported by Pietra et al. [11], also examined somatic JAK2 exon 12 mutations in patients with JAK2 negative myeloproliferative disease. All patients with exon 12 mutations had low erythropoietin levels. In total, eight different types of mutations and two new duplications were found in this study. Napoli and Ignarro [12] described nitric oxide and atherosclerosis, specifically their relationship. The physiological state is maintained in the bloodstream by laminar or turbulent flow. NO is also an inhibitor of blood elements that could bind to the vessel wall. Schwentker et al. [13] tried to answer the question of what role cytokines and nitric oxide play in tissue repair and injury. When NO enzymes are genetically deleted or pharmacologically attenuated, the organism’s wound healing is impaired. They described the roles of NO and cytokines in healing. Vallet [14] discussed vascular reactivity and tissue oxygenation. Injury to the blood circulation may imply a disruption of O2 body regulation, and consequently, pathological restriction may occur.

Myeloproliferative neoplasms are clonal hematopoietic stem cell malignancies characterized by independence or hypersensitivity of hematopoietic progenitors to numerous cytokines. The molecular basis of most myeloproliferative neoplasms is unknown. PV is an acquired myeloproliferative neoplasm, characterized by the presence of polycythemia variously associated with thrombocytosis, leukocytosis, and splenomegaly. PV progenitors are hypersensitive to erythropoietin and other cytokines [15–17]. PV has an incidence rate of 1.0 case per 100,000 people (prevalence rate of 44–57 per 100,000 people) and is a disease of the elderly, with a median age at diagnosis of 61 years. The median survival of PV is 18.9 years. PV has a chronic course, with thrombosis representing a major cause of morbidity and mortality. Less common causes include transformation to myelofibrosis and acute leukemia [18]. When PV is present, determining the peripheral blood hematocrit, or hemoglobin, will not accurately reflect the actual volume of red blood cells in the body because in PV, in contrast to other disorders causing erythrocytosis, the increase in red cell mass is usually associated with an increase in plasma volume [19]. Splenomegaly and myelofibrosis often occur in PV patients. Almost all PV patients harbor a mutation in the JAK2 gene, mainly a JAK2 V617F point mutation [20]. Bone marrow morphology remains the cornerstone of diagnosis. The presence of a JAK2 mutation is expected in PV, while approximately 90% of patients with ET express mutually exclusive JAK2, CALR (calreticulin), or myeloproliferative leukemia mutations [21,22]. PV, ET, and idiopathic myelofibrosis are clonal myeloproliferative neoplasms arising from a multipotent progenitor. The loss of heterozygosity (LOH) on the short arm of chromosome 9 (9pLOH) in myeloproliferative neoplasms suggests that 9p harbors a mutation that contributes to the clonal expansion of hematopoietic cells in these diseases [1]. The molecular pathogenesis of these entities is unknown, but tyrosine kinases have been implicated in several related disorders. The role of the cytoplasmic tyrosine kinase JAK2 in patients with a myeloproliferative disorder has been extensively investigated [3]. PV increases the risk of arterial and venous thromboembolic complications. It was demonstrated that PV is associated with endothelial dysfunction in the preclinical phase of arterial disease [5]. Endothelial dysfunction is a systemic disorder that plays an important role in the pathogenesis of atherosclerosis. Current evidence suggests that endothelial status is determined not only by the individual risk factor burden. Rather, it may be regarded as an integrated index of all atherogenic and atheroprotective factors present in each individual [6]. The laboratory and clinical findings of 179 patients with ET and 77 with PV were classified according to the presence of the JAK2 V617F mutation and compared. The relationship between patients with JAK2 wild-type ET, JAK2 V617F positive ET and PV (all with the JAK2 mutation) was determined. The rate of thrombotic complications in JAK2-positive ET was significantly higher than
in wild-type ET and not statistically different from that of PV patients [8]. The allelic frequency of the JAK2-V617F mutation in DNA and the expression levels of the mutant and wild-type JAK2 mRNA were determined in the granulocytes from 60 patients with ET and 62 patients with PV at the time of diagnosis. Using an allele-specific quantitative polymerase chain reaction (qPCR), JAK2-V617F was detected in 75% of ET and 97% of PV patients at diagnosis [10].

This present paper analyzes anonymized data from a clinical information system. Individuals were screened for high erythrocyte counts in the bloodstream and typical manifestations of PV disease. However, this disease was not detected in them (most of the subjects had the JAKV617F parameter without mutation), so the patients could not be diagnosed with primary myeloproliferative disease.

As summarized in Figure 2, in this work we tested coagulation factors (Factor V Leiden, Factor V R2, Factor II) as well as proteins responsible for the risk of atherosclerosis (ApoB R3500Q, APO E); endothelial ischemia (eNOS-786T>C, eNOS G894T); cardiovascular thrombogenic dysfunction (ACE Ins/Del); metabolic homocysteine thrombogenic dysfunction (MTHFR A1298C, MTHFR C677T); endothelial receptor thromboembolism (EP C4600G, EPCR G4678C); thrombogenic endothelial immunocompetence (LTA C804A); dysfunctional platelet aggregation and adhesion (HPA1 a/b GPIIIa L33P) and protein response during thrombogenesis and thrombolysis (FGB b-fibrinogen −455G>A, Factor XIII, PAI-1 4G/5G).

Figure 2. Investigated parameters within the whole research.
2. Materials and Methods

The starting point of this study was based on a large cohort (approximately 3000 subjects) of active blood donors, who did not demonstrate any clinical symptoms that would exclude them from actively donating blood. This study was approved by the ethical committee of University of Hradec Kralove on 16 August 2021 with the evidence number: 11/2021 (The complete report of the ethical committee can be found in the section: Institutional Review Board Statement). The donors also did not report any medical problems in their questionnaire. Based on transfusion medicine legislation, donors, among other parameters, mandatorily undergo hemoglobin level determination. A total of 190 subjects with repeated hemoglobin levels corresponding to polyglobulia were selected from the initial cohort (182 men and 17 women) and included in the study sample (Figure 3). The men ranged in age from 19 to 65 years and the women from 26 to 61 years. All subjects underwent genetic testing for defined parameters (thrombophilic mutations using real-time PCR, the V614F mutation of the JAK-2 gene–PV, and determination of predispositions to atherosclerosis using PCR and reverse hybridization). This studied cohort of selected donors was typical in its homogeneity, whereby phenotypic signs of the homozygous, heterozygous, or so-called wild-type form, i.e., without mutation, were found in all those included. For the selected subjects, the laboratory hemoglobin levels appeared to be possible signs of myeloproliferative.

![Figure 3. Dependence of age, frequency and sex.](image)

To arrange the blood results in a comprehensible and clear manner, an overall data library was created in Excel. This was later imported into MATLAB R2019a, where data analysis with evaluation of individual parameter frequency was performed. Typified groups were thus created, and parameters with similar properties were unified. Data mining was used to process the data, whereby a contingency table of all possible mutation combinations was acquired. Parameters with a value close to zero were excluded as clinically nonsignificant. All parameters were then divided according to their correlations and their frequency, i.e., incidence, into three groups (without mutation, mutation at a ratio of 1:1 and predominantly heterozygous detection). Two hypotheses were defined as follows: the combination of homozygous and heterozygous predispositions carry a clinically more
significant risk than a homozygous predisposition; homozygous predispositions carry a clinically higher risk than heterozygous predispositions.

2.1. Instrumentation for Analysis

Genetic predispositions to atherosclerosis were determined with the help of reverse hybridization, using CVD Strip Assay commercial kit (ViennaLab Diagnostics GmbH, Austria) and Applied Biosystems™ ProFlex™ PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA). Genetic testing of predisposition to venous thrombosis was based on the method of allelic discrimination TaqMan™ SNP genotyping Assays (Thermo Fisher Scientific Inc., Waltham, MA, USA) and Applied Biosystems™ 7300 Real-Time PCR System (Thermo Fisher Scientific Inc., Waltham, MA, USA). The V617F mutation in the JAK2 gene was determined using Realquality RS-JAK-2 V617F (AB Analitica®, Padua, Italy) and Applied Biosystems™ 7300 Real-Time PCR System.

2.2. Investigated Parameters

2.2.1. Risk of Developing Atherosclerosis—ApoB: Familial Defective Apolipoprotein B-100; ApoE

Apolipoprotein B-100 is one of the forms of apolipoprotein B (ApoB). It represents the basic protein component of low (LDL) and very low (VLDL) lipoproteins. It plays an important role in the transport of lipids and cholesterol. The R3500Q mutation is one of the most widespread mutations involving the ApoB gene [23]. It is caused by the substitution of the amino acid base G (guanine) for A (adenine) in exon 26. This leads to the substitution of arginine with glutamine at position 3527, leading to a change in the protein structure at the site of receptor binding. This results in a lower affinity of LDL particles to receptors and their subsequent accumulation in the blood. The familial genetic defect of ApoB is one of the causes of familial hypercholesterolemia. Accumulation of cholesterol in the blood significantly increases the risk of atherosclerosis and myocardial infarction. The frequency of heterozygotes is 1:500, and the frequency of mutated homozygotes is 1:1,000,000 [24,25].

Apolipoprotein E (ApoE) is a component of VLDL, and its main task is to remove excess cholesterol from the blood and transport it into the liver for further processing [26]. It ensures that cholesterol levels remain physiological and thus significantly participates in the prevention of cardiovascular disease. Three commonly occurring alleles of the ApoE gene have been identified—ApoE2, ApoE3 and ApoE4. The E2/E2 homozygote is exposed to a higher risk of familial hyperlipoproteinemia (type III). In contrast, the ApoE4 isoform is a factor for atherosclerosis because of the increased level of total cholesterol, both in the heterozygous and especially in the homozygous form. The E4 allele has been identified as a significant and independent (on age, gender, and atherosclerotic disease) predictive factor for the development of aortic stenosis [27,28].

2.2.2. Coagulation Factors and Their Mutations—Factor V Leiden, Factor V R2, Factor II

Mutations of coagulation factors are often associated with a higher risk of thrombophilia. Under physiological conditions, the coagulation cascade ensures the formation of blood clots. Factor V Leiden (G1691A) is the most common genetic predisposition for thrombosis. This point mutation induces resistance to the anticoagulant activity of APC (activated protein C) in factor V, and this consequently prolongs the process of thrombosis. The Leiden mutation is the most frequent congenital predisposition for abnormal blood clotting. Heterozygotes are more frequent in the population than homozygotes. The risk of developing thromboembolism is 3–8 × higher in heterozygotes than in the unmutated population, and it is 50–80 × higher in homozygotes [29–31]. Factor V R2 (H1299R) is another genetic mutation of factor V. It is induced by polymorphism A4070G in exon 13, leading to the substitution of histidine (R1 allele) for arginine (R2 allele) at 1299 B of the domain. This substitution leads to a decrease in factor V levels and its resistance to activated protein C (APC) [32]. Factor II prothrombin (G20210A) is one of the most frequent genetic thrombophilic predispositions in the European population and plays a significant role in
venous thrombosis. Together with factor V, prothrombin is part of the coagulation cascade ensuring correct blood clotting [33]

2.2.3. Endothelial Ischemia—eNOS (−786T>C), eNOS (G894T)

Endothelial nitric oxide synthase (eNOS) is one of the three isoforms of nitric oxide (NO) synthesis that demonstrate sequence and functional homology. The gene for eNOS (that is, NOS3) is located on chromosome 7q35-36. NO formation is catalyzed by endothelial NO synthase. It has vasodilating, anti-inflammatory, and antiproliferative properties. Decreased production of NO may, among others, lead to smooth muscle cell proliferation. Moreover, eNOS (G894T) polymorphism for the NO synthase gene has been associated with the incidence of coronary artery spasms and myocardial infarction and in some cases has been a predictor of restenosis. In contrast, eNOS (G894T) polymorphism in the same gene is a predictor of serious coronary events without increasing the risk of in-stent restenosis. Similarly, another polymorphism in the promoter region of the NOS3 gene (−786T>C) affects the expression of this gene, increasing susceptibility to coronary disease [34–36].

2.2.4. Cardiovascular Thrombogenic Dysfunction (ACE Ins/Del)

Angiotensin converting enzyme (ACE) plays an important role in the regulation of blood pressure as part of the renin–angiotensin system. ACE is present on the endothelial cells in many tissues (e.g., the uterus, placenta, heart, brain, kidneys, leukocytes, alveolar macrophages, peripheral monocytes and neurons). It enables the conversion of angiotensin I to the vasoconstrictive-aldosterone-stimulating peptide angiotensin II (ATII) which participates in vasoconstriction [37,38].

2.2.5. Metabolic Homocysteine Thrombogenic Dysfunction—MTHFR A1298C, MTHFR C677T

The 5,10-methylenetetrahydrofolate reductase (MTHFR) enzyme is a key enzyme for the metabolism of homocysteine. Point mutations in the MTHFR gene lead to the formation of an enzyme with increased thermolability and decreased activity, and this significantly correlates with an increased level of homocysteine in plasma. Homocysteine may thus contribute to the development of atherosclerosis and thrombosis because of changes in vascular cell proliferation and promotion of prothrombotic activities within the vascular wall. The most frequent and best-known mutations of the MTHFR gene involve substitution of C for T at position 677 (C677T). The second most frequent mutation involves the substitution of A for C, at position 1298 (A1298C). Approximately 30% of homozygotes and 65% of heterozygotes with the C677T mutation demonstrate MTHFR activity. The A1298C mutation in both the homozygous and heterozygous form, per se, does not significantly alter plasma levels. However, a compound heterozygote with C677T becomes a risk factor for milder hyperhomocysteinemia [39,40].

2.2.6. Endothelial Receptor Thromboembolism—EPCR A4600G, EPCR G4678C

EPCR is an endothelial protein C receptor predominantly located on the endothelium of large vessels. Its physiological role consists of localizing protein C in order to be activated by the thrombin–thrombomodulin complex. It occurs as the A3 haplotype (A4600G) and the A1 haplotype (G4678C). Individuals with the A3 haplotype have higher plasma levels of EPCR and are thus at risk of venous thrombosis. In contrast, a protective effect against venous thromboembolism may manifest in individuals with the A1 haplotype [41,42].

2.2.7. Thrombogenic Endothelial Immunocompetence—LTA C804A

Lymphotoxin-alfa (LTA) is a representative of the tumor necrosis family of cytokines and was originally isolated on the basis of its antitumor activity. Later on, its anti-inflammatory and immunological activities were demonstrated. The LTA gene is located on chromosome 6p21.3. LTA as an inflammatory cytokine is expressed in atherosclerotic lesions, and it plays a leading role in the development of atherosclerosis (the greatest risk factor
for arterial accidents). In this context, an association has been found between the C804A (T26N) polymorphism for the LTA gene and coronary and cerebrovascular accidents [43].

2.2.8. Dysfunctional Platelet Adhesion and Aggregation—HP A1 a/b GPIII L33P

The HP A1 (a/b)–GPIIIa (L33P)–platelet glycoprotein complex IIb/IIIa (a2bb3) plays a role in cell interactions and includes binding sites for fibrinogen, the von Willebrand factor, fibronectin and vitronectin. Polymorphisms in the genes coding these glycoprotein complexes may affect many processes in the human body (resistance to aspirin, cardiovascular disease, changes in molecule antigenic properties possibly leading to posttransfusion purpura, life-threatening thrombocytopenia and neonatal thrombocytopenic purpura). The most common and clinically significant alleles of the GPIIIa gene are P1A1 (HP A-1a) and P1A2 (HP A-1b). HP A-1b may in some cases increase platelet aggregation. Heterozygotes and homozygotes for HP A-1b more frequently develop thin-walled, vulnerable atherosclerotic plaques, prone to ruptures and subsequent massive thrombosis. However, carriers of the HP A-1b allele only have a mild risk of developing coronary artery disease, myocardial infarction, or restenosis following percutaneous interventions. Nonetheless, this risk increases significantly with the concomitant incidence of other polymorphisms such as, e.g., those for the eNOS and PAI-1 genes. However, the presence of at least one allele of HP A-1b is currently considered to be one of the thrombophilic factors for pre-eclampsia in pregnant women [44,45].

2.2.9. Thrombogenesis and Thrombolysis—FGB b-fibrinogen—455G>A, Factor XIII, PAI-1 4G/5G

FGB b-fibrinogen (−55G>A) is the coagulation factor I with a molecular weight of 340 kDa. This dissolvable glycoprotein is commonly found in blood plasma as well as in platelet granules. It is one of the key proteins of thermocoagulation. It plays a role in platelet aggregation and affects plasma viscosity. The presence of the A-allele (with an incidence of approximately 20% in the population) is associated with a significantly increased promoter activity of the gene and thus with increased fibrinogen plasma levels. The −455G>A polymorphism is significant in relation to fibrinogen levels in persons with coronary artery disease as well as in patients after revascularization bypass surgery [46]. Factor XIII (V34L), or fibrin-stabilizing factor, is a transglutaminase consisting of a tetramer of two A and two B units. The nucleotide substitution of amino acid bases C (cytosine) for T (thymine) in exon 2 of the F13A1 gene to the substitution of valine for leucine at position 34 of the peptide chain. Homozygotes for this mutation demonstrate a significantly higher activity of this enzyme than individuals without any mutation, while heterozygotes demonstrate an intermediate activity of this enzyme. It has been demonstrated that the Lue 34 variant has an important protective effect against venous thromboembolism [47]. PAI-1 4G/5G is a key inhibitor of tissue plasminogen activator (tPA) and urokinase (uPA) as well as of plasminogen activators and consequently of fibrinolysis. The PLANH1 promoter gene coding for PAI-1 may include a polymorphism known as 4G/5G whereby the 5G allele is less transcriptionally active than the 4 G allele. Primary elevation of PAI-1 levels is found in individuals with 4G/4G polymorphism. It is assumed that this polymorphism is associated with a higher incidence of arterial thrombosis [48].

3. Results

Results of clinical evaluation for the studied subjects are summarized in the following Tables 1 and 2.

The parameters were then divided according to the frequency of mutation incidence expressed in a percentage (heterozygote and homozygote). Thanks to this distribution, we were able to organize parameters into groups in which they occurred most frequently.

Men of all ages predominated in the studied sample. In most individuals, the primary myeloproliferative neoplasm was not confirmed. This means that the subjects in this sample suffered from secondary PV.
Table 1. Percentage evaluation of results in the table.

| <20% with Mutation | 20–50% with Mutation | >50% with Mutation |
|--------------------|----------------------|--------------------|
| JAK V617F          | eNOS G894T           | eNOS-7867>C        |
| Apo B R3500Q       | LTA C804A            | ACE Ins/Del        |
| Factor V Leiden    | HPA1 a/b GPIIla L33P | FGB b-fibrinogen –455G>A |
| Factor V R2        | Factor XIII          | MTHFR C677T        |
| Factor II          | EPCR A4600G          | EPCR G4678C        |
| -                  | Apo E                | MTHFR A1298C       |
| -                  | -                    | PAI-1 4G/5G        |

Table 2. Percentage evaluation of results in the table after distribution.

| <20% with Mutation | 20–50% with Mutation | >50% with Mutation |
|--------------------|----------------------|--------------------|
| Primary PV         | Endothelial ischemia | Endothelial ischemia |
| Dyslipidemia-atherosclerosis | Thrombogenic endothelial immunocompetence | Cardiovascular thrombogenic dysfunction |
| Thrombogenicity of clotting factors | Dysfunction of platelet aggregation and adhesion | Thrombogenesis and thrombolysis |
| Thrombogenicity of clotting factors | Thrombogenesis and thrombolysis | Metabolic homocysteine thrombogenic dysfunction |
| Thrombogenicity of clotting factors | Receptor endothelial thromboembolism | Receptor endothelial thromboembolism |
| -                  | Dyslipidemia-atherosclerosis | Metabolic homocysteine thrombogenic dysfunction |
| -                  | -                     | Thrombogenesis and thrombolysis |

It was thus inferred that this was not the case of a primary myeloproliferative disease (given that the sample consisted of secondary nonautonomous PV). The last parameter was that of dyslipidemia–atherosclerosis involving the Apo B R3500Q genotype. The parameter involving the Apo E genotype occurred in the 20 to 50% group. It was thus inferred that the parameter was not clinically relevant for our needs. The 20 to 50% group included the parameter of platelet aggregation and adhesion dysfunction, i.e., HPA1 a/b GPIIla L33P. This parameter was projected to occur in the group of greater than 50%.

The group with less than 20% incidence included coagulation factor thrombogenicity. This parameter occurred solely in the group with less than 20% incidence, and thus appeared to be clinically nonrelevant (Figures 4 and 5). Coagulation factor thrombogenicity integrates factor V Leiden, factor V R2 and factor II. This group also includes primary JAK V617F positive PV, which was demonstrated in only two subjects within the entire sample. The question thus arises as to whether it is necessary to influence platelet function using antiplatelet agents or vice versa. The 20 to 50% group also included the thrombogenic endothelial immunocompetence LTA C804A parameter. It occurred in subjects at a ratio of 1:1 for heterozygosity and nonmutated allele, i.e., wild type, similar to factor XIII thrombogenesis and thrombolysis (Figure 6).
Figure 4. Clearly negative mutation capture. Group of clotting factor Thrombogenicity of is evaluated.

Figure 5. Clearly negative mutation capture. Here is dyslipidemia-atherosclerosis and receptor of endothelial thromboembolism \textit{EPCR A4600G}. 
Figure 6. Heterozygous distribution and nonmutant alleles in a 1:1 ratio.

The parameter of endothelial receptor thromboembolism, i.e., EPCR A4600G, demonstrated unequivocally negative detection of the mutation. A 50% risk of mutation incidence was inferred based on the results of the 20 to 50% group. The question remains whether testing for these parameters clinical significance has, given that the 1:1 ratio will be less contributing than in the greater than 50% group. Individuals with a 50% incidence of mutations harbored endothelial ischemia and thrombogenesis and thrombolysis, i.e., FGB b-fibrinogen, at a ratio of 1:1 for heterozygosity and nonmutated allele.

Other representative parameters of the thrombogenesis and thrombolysis, i.e., PAI-1 4G/5G and endothelial receptor thromboembolism, i.e., EPCR G4678C, were dominant on heterozygote detection (Figure 7).

Figure 7. Dominating heterozygosity.
One important finding was that the greater than 50% group included cardiovascular thrombogenic dysfunction $ACE\ ins/del$, which had not been given much attention previously.

The last parameter was the metabolic homocysteine thrombogenic dysfunction, i.e., $MTHFR$, which occurred at a ratio of 1:1 for heterozygosity and nonmutated allele (Figure 8).

![Figure 8. Heterozygous distribution and nonmutant alleles in a 1:1 ratio.](image)

Interestingly, both studied $MTHFR$ parameters were found only in the group with a greater than 50% incidence. In this group, the 1:1 distribution was clinically more significant than in the 20 to 50% group. It is possible to derive from these tables that the greater than 50% group, which includes cardiovascular thrombogenic dysfunction and metabolic homocysteine thrombogenic dysfunction are the most significant from a clinical aspect. This is followed by the 20 to 50% group. In the less than 20% group, endothelial thromboembolism, thrombogenesis and thrombolysis appear to be most significant.

3.1. Evaluation of the Group with Detection Less than 20%

The group with an incidence of less than 20% includes parameters such as primary $JAK\ V617F$ positive $PV$, dyslipidemia–atherosclerosis and coagulation factor thrombogenicity. All these parameters appeared indeterminate in the analysis of the data. Based on results, it was not possible to determine whether it was a case of direct or indirect correlation. For this reason and because of their low incidence, these parameters were evaluated only marginally, and they appear nonsignificant for the clinical interpretation of this study.

3.2. Evaluation of the Group with Detection from 20 to 50%

The $APO\ E$ parameter (dyslipidemia-atherosclerosis) in the $E3/E3$ allele demonstrated indirect dependency for $eNOS\ G894T=homozygote$ (endothelial ischemia) as well as for $EPCR\ A4600G=homozygote$ (endothelial receptor thromboembolism), factor $XIII=homozygote$ (thrombogenesis and thrombolysis), $HPA1\ a/b\ GPIIIa\ L33P=a/b$ (dysfunction of platelet aggregation and adhesion) and $LTA\ C804A=homozygote$ (thrombogenic endothelial immuno-competence). The $APO\ E=E2/E2$ parameter demonstrated a direct correlation with factor $XIII=homozygote$, which has a protective effect. The $eNOS\ G894T$ parameter in the homozygous allele was in direct correlation only with factor $XIII=homozygote$, which reduces risk
as homozygous factor XIII has a protective effect. The eNOS G894T in the nonmutated allele was indirectly dependent on Apo E=E2/E2, HPA1 a/b GPIIIa L33P=a/b (heterozygote) and LTA C804A=homozygote (thrombogenic endothelial immunocompetence). The EPCR A4600G=homozygote parameter was in direct correlation with eNOS.

The presence of G894T=homozygote and LTA C804A=homozygote indicate an acceleration risk. The EPCR A4600G parameter in the nonmutated wild-type allele was indirectly dependent on APO E=E2/E2, homozygote eNOS G894T, Factor XIII, LTA C804A and HPA1 a/b GPIIIa L33P=a/b (heterozygote).

Factor XIII was in direct correlation with eNOS G894T=homozygote and LTA C804A=homozygote (risk reduction). For the nonmutated and heterozygote allele, the parameter was indirectly correlated with homozygous EPCR A4600G. The HPA1 a/b GPIIIa L33P=a/a parameter was indirectly dependent on Apo E=E2/E2 and the homozygous allele of eNOS G894T, EPCR A4600G, Factor XIII and LTA C804. Detected heterozygotes were in direct correlation with homozygous factor XIII, again reducing risk.

The last studied parameter in this group was LTA C804A. Homozygosity was in direct correlation with homozygous factor XIII. The nonmutated allele was indirectly dependent on Apo E=E2/E2, homozygote eNOS G894T and heterozygous HPA1 a/b GPIIIa L33P.

3.3. Evaluation of the Group with Detection Greater than >50%

The ACE ins/del=ins/del parameter (cardiovascular thrombogenic dysfunction) was significant when detected in the heterozygous form, where it was shown be indirectly dependent on eNOS −786T>C=homozygote (endothelial ischemia) and FGB b-fibrinogen 455G>A=homozygote (thrombogenesis and thrombolysis). If the ACE parameter is in the heterozygous form, the risk of endothelial ischemia and thrombogenesis and thrombolysis decreases. It was also shown that this ACE ins/del=ins/ins parameter was directly dependent on EPCR G4678C=homozygote (endothelial thromboembolism receptor), MTHFR A1298C=homozygote, MTHFR C677T=homozygote and PAI-1 4G/5G=5G/5G. In this case, we refer to an acceleration risk. The eNOS −786T>C=heterozygote parameter (endothelial ischemia) was significant in the case of heterozygosity, which was shown to be directly dependent on ACE ins/del=ins/del (cardiovascular thrombogenic dysfunction) and FGB b-fibrinogen −455G>A=homozygote (thrombogenesis and thrombolysis). The homozygous form of eNOS −786T>C was also shown to be directly dependent on EPCR G4678C, MTHFR A1298C, MTHFR C677T and PAI-1 4G/5G, all in the homozygous form. The eNOS parameter in the homozygous form increases probability in the group of metabolic homocysteine thrombogenic dysfunction. The EPCR G4678C parameter (receptor endothelial thromboembolism) in the heterozygous form was indirectly correlated to MTHFR A1298C=homozygote and MTHFR C677T=homozygote (metabolic homocysteine thrombogenic dysfunction) as well as PAI-1 4G/5G=5G/5G (thrombogenesis and thrombolysis). The homozygous form of EPCR G4678C was significant because of its direct correlation with ACE ins/del=ins/dels, as well as homozygous eNOS −786T>C and FGB b-fibrinogen −455G>A. The FGB b-fibrinogen −455G>A parameter in its homozygous form was in direct correlation with homozygous EPCR G4678C, MTHFR A1298C and C677T as well as PAI-1 4G/5G=5G/5G. The wild-type allele of fibrinogen was then indirectly correlated to ACE ins/del=ins/dels and homozygous eNOS −786T>C. The MTHFR A1298C homozygous parameter was directly correlated with ACE ins/del=ins/dels, eNOS −786T>C=homozygote and FGB b-fibrinogen −455G>A=homozygote. Heterozygous MTHFR A1298C was indirectly correlated with homozygous EPCR G4778C, MTHFR C677T and PAI-1 4G/5G. The MTHFR C677T=homozygote parameter was directly correlated with ACE ins/del=ins/dels (this result was highly significant for cardiac risk), eNOS −786T>C=homozygote and FGB b-fibrinogen455G>A=homozygote. MTHFR C677T heterozygote demonstrated an indirect correlation with homozygous EPCR G4778T, MTHFR A1298C and PAI-1 4G/5G=5G/5G. The PAI-1 4G/5G=4G/5G was indirectly correlated with the homozygous allele of EPCR G4778C, MTHFR A1298C, MTHFR C677T and PAI-1 4G/5G=5G/5G. The homozygous PAI-1 allele was directly correlated to ACE ins/del=ins/dels, homozygous eNOS −786T>C and FGB b-fibrinogen −455G>A.
4. Discussion and Conclusions

This research aimed to analyze the results of genetic thrombogenic and atherogenic parameters in selected donors (predominantly men) who had higher levels of hemoglobin but no diagnosis of the chronic progressive myeloproliferative disease, PV. This disease predominantly involves mutations of the JAK V617F gene. Data were acquired from Nový Jičín Hospital, in the Czech Republic. The study sample was a homogenous group of 199 patients aged between 19 and 65 years who had high levels of hemoglobin and physical symptoms of PV, and in whom a primary myeloproliferative disorder had not been demonstrated.

The most contributive groups included endothelial ischemia, cardiovascular thrombogenic dysfunction, metabolic homocysteine thrombogenic dysfunction, thrombogenesis and thrombolysis and endothelial receptor thromboembolism. All the parameters in these groups were clinically relevant. The ACE ins/del parameter and cardiogenic risk demonstrated great significance. This enzyme, usually synthesized by endothelial cells, regulates blood pressure (smooth muscle constriction and subsequent arterial narrowing). If ACE was present in the heterozygous form, the thrombogenic risk in endothelial ischemia, as well as thrombogenesis and thrombolysis, decreased. In the homozygous form, the risk increased in relation to the parameters MTHFR, EPCR G4678C and PAI-1 (all in the homozygous form). Clinically relevant parameters were detected in the endothelial ischemia group. The parameters studied included eNOS G894T and eNOS –786T>C. Both these oxides have vasodilating, anti-inflammatory and antiproliferative properties. Mutations involving these parameters lead to smooth muscle proliferation, which may lead to coronary artery disease. The eNOS –786T>C parameter in the homozygous form increased the risk in the case of homocysteine. Furthermore, homozygosity for eNOS G894T, EPCR A4600G and LTAC804A demonstrated an acceleration risk. Metabolic homocysteine thrombogenic dysfunction was the only one to occur in the greater than 50% group. Mutations of these genes lead to changes in DNA that subsequently increase blood homocysteine levels, leading to cardiovascular disease or thrombosis. MTHFR A1298C in combination with MTHFR C677T was a risk factor. The MTHFR C677T homozygote parameter with ACE ins/ins represented a major cardiogenic risk. Endothelial receptor thromboembolism EPCR occurred in the 20 to 50% group as well as in the greater than 50% group. This endothelial receptor most frequently occurs on the vascular endothelium. EPCR A4600G in its homozygous allele carried an acceleration risk with eNOS G894T and LTA C804A. Conversely, EPCR G4678C clinically decreased the inference of thrombogenic risk in the group of metabolic homocysteine thrombogenic dysfunction. The last parameter was PAI-1 4G/5G, a regulator of homeostasis that inhibits the plasminogen activator. The whole study included 52% of heterozygotes and 16% of homozygotes for this gene. Homozygosity increased the risk in relation to cardiovascular thrombogenic dysfunction, endothelial receptor thromboembolism and homocysteine thrombogenic dysfunction. ACE ins/del (cardiovascular thrombogenic dysfunction) appeared to be an unequivocal parameter, one that was mutated in all groups and thus carried an acceleration risk. Based on the results, groups with a higher incidence of mutations were assessed as presenting a greater risk for patients with regards to PV. Among the studied patients, it was not possible to determine unequivocally who had a higher risk of developing this disease, as the individual combinations of mutations could manifest as different symptoms of the disease. In general, however, it was possible to state that the whole studied sample was at risk in the presence of manifestations of PV and no unequivocal diagnosis. The group of less than 20% incidence appeared to be clinically nonsignificant for the PV investigation and therefore not testing irrelevant mutations could prove cost-effective. Conversely, the JAK V617F parameter from this group must be tested, as it can unequivocally rule out or confirm PV as the primary disease.

The above results obtained through the present study helped to select appropriate parameters for the prediction of myeloproliferative disease, which has not been previously genetically confirmed in patients who show signs of this disease. Research in this area will be continued and a statistical sample of patients with signs of the disease will be expanded.
on which to validate selected parameters that could be used to detect the disease. Further extensive meta-analysis will be performed on these data samples.

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**Informed Consent Statement:** All the patient’s records were anonymized. All the patients who are involved in this study have signed an informed consent before medical examination that they agree with including their medical records in this study for research purposes.

**Data Availability Statement:** All the data, which are used in this study can be found in the link: https://www.dropbox.com/sh/s5vs8eh2wrwqogo/AAAXZs6lkjpjBTvC1XNwrw2Wa?dl=0.

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