RESEARCH ARTICLE

Occurrence of the *JAK2 V617F* mutation in patients with peripheral arterial disease

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The acquired *JAK2 V617F* mutation is common in patients with myeloproliferative neoplasms. We previously showed that *JAK2 V617F* is also found in coronary patients, most of them affected by coronary atherosclerosis. Peripheral arterial disease (PAD) is another important manifestation of atherosclerosis. However, prevalence of the *JAK2 V617F* mutation and its effect on clinical or hematologic characteristics is unknown in PAD patients. In the present study we determined the prevalence of *JAK2 V617F* in a cohort of 287 patients with sonographically proven PAD and compared mutation frequency with mutational status of 997 healthy people from the KORA F4 study. *JAK2 V617F* screening and quantification of allele burden in both cohorts was performed with same allele-specific quantitative real-time PCR method. From a total of 287 PAD patients, 9 individuals were tested positive for the *JAK2 V617F* mutation. One patient showed elevated hemoglobin values, indicating polycythemia vera. Observed *JAK2 V617F* frequency (3.1%) in PAD patients showed a 5-fold, highly significant increase compared with healthy people ($P < 0.001$). Furthermore, occurrence of the mutation in PAD patients was significantly decreased in patients using aspirin ($P = 0.003$). We conclude that the prevalence of *JAK2 V617F* mutation is significantly increased in PAD patients compared to the general population. Future studies are warranted to confirm our observations and to define the underlying mechanisms behind our findings.

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Introduction

Peripheral artery disease (PAD) is one of the most common manifestations of atherosclerosis and PAD patients are at exceptionally high risk for cardiovascular morbidity and mortality [1], showing a worse prognosis than that of patients with coronary artery disease (CAD) [2].

Inflammation plays a crucial role in the initiation and progression of PAD and the inflammatory mediators involved in this process are similar to those contributing to the development of CAD [3–7]. Smoking and type 2 diabetes mellitus are the strongest predictors of developing PAD and promote oxidative stress, which directly or indirectly enhances inflammatory pathways [3,8].

The Janus kinase 2 (JAK2)/signal transducer and activator of transcription (STAT) pathway is a critical regulator of inflammatory processes, transmitting extracellular signals from cytokines and growth factors to the nucleus, thus activating or repressing transcription of target genes [9,10]. Once activated, the JAK/STAT pathway stimulates cell proliferation, differentiation, migration, and apoptosis critically involved in growth control, and therefore, represents a crucial pathway implicated in promoting tumorigenesis [9–11]. Due to the close relation between inflammation and atherosclerosis [3,6,7], the JAK/STAT pathway has also been linked to atherogenesis and the manifestation of vascular disease [12–14].

An acquired mutation within the *JAK2* gene, an amino acid substitution of valine-to-phenylalanine at position 617 (V617F), disrupts the auto-inhibition of JAK2 and results in constitutive activation of the JAK2/STAT signalling pathway [15–17]. The mutation is common in patients with Philadelphia-negative myeloproliferative neoplasms (MPNs), a group of rare clonal hematopoietic stem cell disorders, including polycythemia vera (PV) and essential thrombocythemia (ET). Here, the *JAK2 V617F* mutation is detected in more than 95% of PV patients and in approximately 50% of patients with ET [18,19]. The presence of the *JAK2 V617F* mutation clearly identifies the process as neoplastic [15–17,20] and, thus screening for JAK2 V617F mutational status has been included in the diagnostic criteria of the World Health Organization (WHO) classification for PV and ET [21].

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TABLE I. Peripheral Arterial Disease Patients' Characteristics with Respect to JAK2 V617F Mutation Status

	JAK2 V617F status		
	Negative (n = 278)	Positive (n = 9)	P-value
Male sex	202 (72.7%)	8 (88.9%)	0.280
Age (years)	66.7 ± 9.9	70.4 ± 8.1	0.278
BMI (kg/m^2)	27.0 ± 4.4	25.5 ± 3.8	0.299
History of smoking, n	238 (85.6%)	8 (85.9%)	0.782
Hypertension, n	227 (84.4%)	8 (88.9%)	0.713
T2DM, n	91 (32.7%)	4 (44.4%)	0.462
Coronary artery disease, n	93 (34.1%)	4 (50.0%)	0.350
Prior myocardial infarction, n	55 (19.8%)	4 (44.4%)	0.072
Prior cerebrovascular events, n	38 (13.8%)	0 (0.0%)	0.243
Prior surgical coronary intervention, n	133 (48.5%)	6 (66.7%)	0.284
Aspirin, n	191 (68.7%)	2 (22.2%)	0.003
Clopidogrel, n	78 (28.1%)	2 (22.2%)	0.701
ACE inhibitors or AT2 antagonists, n	164 (59.0%)	4 (44.4%)	0.383
Statins, n	192 (69.1%)	4 (44.4%)	0.118
WBC (10^9/L)	7.4 ± 2.2	7.7 ± 2.3	0.695
RBC (10^{12}/L)	4.6 ± 0.6	4.9 ± 0.4	0.161
HGB (g/L)	140 ± 18	145 ± 20	0.410
HCT (%)	42 ± 5	44 ± 6	0.362
PLT (10^9/L)	254 ± 123	277 ± 65	0.264
CRP (mg/dl)	1.00 ± 1.87	0.56 ± 0.55	0.944

T2DM, type 2 diabetes mellitus; ACE, angiotensin converting enzyme; AT2, angiotensin-2; WBC, white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; PLT, platelets.
Differences in continuous variables and categorical study variables were tested for statistical significance with the t-test and with the Chi-square test, respectively.

Occurrence of the *JAK2 V617F* mutation has also been reported in individuals from the general population [22–25] or hospitalized patients [26,27] in the absence of signs of MPN with variable frequencies ranging between 0.2% and 15%. This discrepancy between various studies mostly depends on the sensitivity of used screening methods to detect the mutation [26,28]. Interestingly, in a cohort of hospitalized smokers and nonsmokers, *JAK2 V617F* mutation was found to be more prevalent among smokers than nonsmokers [26]. Furthermore, we reported recently a prevalence of 1.32% (21/1,589) of the JAK2 V617F mutation in coronary patients, including a high proportion of patients with angiographically proven CAD [28]. Frequency of the mutation was more than two-times increased in coronary patients compared with sex- and age-matched healthy controls, but difference did not reach statistical significance.

The *JAK2 V617F* mutation may be accumulated in PAD patients as well, potentially contributing to the manifestation of the disease via constitutive activation of the JAK2/STAT signalling pathway extending inflammatory response or, vice versa, induced by risk factors of PAD, leading to an increased rate of inflammation and oxidative stress [3,26,29,30]. So far, frequency of JAK2 V617F in PAD patients and a possible correlation between characteristics of PAD patients and *JAK2 V617F* mutational status is unknown. Therefore, we aimed to investigate *JAK2 V617F* mutation status in a cohort of PAD patients and its impact on blood cell counts and other clinical or biochemical characteristics. Furthermore, we compared *JAK2 V617F* mutation frequency between PAD patients and subjects from the general population with a negative medical history of atherothrombotic disease, to elucidate a possible correlation of JAK2 V617F mutation prevalence with PAD.

Methods

Study subjects. This study includes 287 PAD patients, who were referred for the evaluation of established or suspected PAD to the Angiology Clinic at the Academic Teaching Hospital Feldkirch, a tertiary care centre in Western Austria (state of Vorarlberg). PAD was defined as any sonographically detectable atherosclerosis in peripheral arteries [31]. For ultrasound examination, we used a Philips iU22 ultrasound system. PAD was diagnosed by direct visualization of atherosclerotic plaques in peripheral arteries of the lower limbs. The scanning protocol included a completed lower limb sonography. All patients in the PAD group had at least one stenosis of more than 50% of at least one of these arteries. Collection of clinical and biochemical characteristics of these patients were described in detail previously [32].

DNA samples of 997 subjects from the general population and with a negative medical history of PAD and CAD served as controls and were obtained from participants of the "Cooperative Health Research in the Region Augsburg" (KORA) F4 study. The KORA study is an independent population-based sample from the general population living in the region of Augsburg, Germany. Details about the KORA study were already described elsewhere [33,34].

Diagnosis of MPN. Diagnosis of PV and ET, respectively, was made according to WHO criteria [21], based on JAK2 V617F mutation status and peripheral blood counts: Diagnosis of PV was made in the presence of JAK2 V617F mutation and elevated hemoglobin $>$ 185 g/L in men or $>$ 165 g/L in women. Diagnosis of ET was done in the presence of JAK2 V617F mutation and elevated platelets $\geq$ 450 $\times$ 10^9/L.

Genotyping. Genomic DNA was extracted from EDTA blood using the peqGOLD® Blood DNA Mini kit (PEQLAB Biotechnologie, Erlangen, Germany) for the PAD patients and using the PuregeneTM DNA Isolation Kit (Gentra Systems, MN 55441) for the KORA F4 study. Screening for the *JAK2 V617F* mutation was carried out by allele-specific real-time PCR as described previously [28]. Samples found positive for the *JAK2 V617F* mutation, copy numbers of mutant and wild-type alleles were quantified using the JAK2 MutaQuant^{TM} assay (Ipsogen, Marseille, France) according to the manufacture's protocol. Percentage of mutant JAK2 V617F alleles was calculated as the ratio of copy number of mutant JAK2 V617 alleles to total copy number of JAK2 alleles (wild-type and mutant). All allele-specific real-time PCRs were carried out on a LightCycler® 480 Real-Time PCR System (F. Hoffmann-La Roche, Basle, Switzerland).

Statistics. Differences in categorical study variables were tested for statistical significance with the Chi-square test and multivariate regression analysis. Differences in continuous variables (age and blood counts) between patients with and without *JAK2 V617F* mutation were tested for statistical significance with the t-test. The Kolmogorov-Smirnov test was used as a test for normality. Non-normally distributed variables (i.e., WBC, hemoglobin, hematocrit, and platelets as well as *JAK2 V617F* mutant percentage) were log-transformed prior to statistical analysis. The distribution of continuous variables is given as mean ± SD (of non log-transformed values). Statistical analyses were performed with the software package SPSS 11.0 for Windows (SPSS, Chicago, IL). Statistical significance was defined as a two-tailed P value $<$ 0.05.

Ethic issues. This study was carried out in accordance with the principles of the Declaration of Helsinki; all participants gave written informed consent. The Ethics Committee of the Medical University of Innsbruck, Austria, approved this study.

Results

Overall, among our 287 PAD patients, there was a preponderance of male gender (73.2%), and a high prevalence of a history of smoking (85.7%), T2DM (33.1%), and hypertension (84.5%). Nine patients were found positive for the *JAK2 V617F* mutation (3.1%). Patients' characteristics with respect to *JAK2 V617F* mutation status are given in Table I. PAD patients on aspirin therapy showed a significantly declined *JAK2 V617F* mutation rate (P = 0.003). Further clinical and biochemical characteristics (including blood cell counts) of our patients were not significantly associated with *JAK2 V617F* mutational status.

Individual patients' blood values from *JAK2 V617F* positive patients are given in Table II. Median mutant allele burden was 0.75%, ranging between 0.2% and 96.2. The patient with the highest mutant allele burden (n°140) showed elevated hemoglobin values, indicating PV.

Selected participants from the KORA study showed a mean age of 66.8 (±6.5) years and a preponderance of male gender (66.1%). Frequency of the *JAK2 V617F* mutation of included participants of the KORA F4 study was 0.61% (6/997), with a median mutant allele load

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TABLE II. Individual Patients' Blood Values and Diagnosis of MPN

Patient-ID	Sex	Age (years)	WBC (10^9/L)	RBC (10^{12}/L)	HGB (g/L)	HCT (%)	PLT (10^9/L)	JAK2 V617F (%MA)	Diagnosis of MPN
26	m	60.1	9.2	4.87	152	45	255	0.84	-
39	m	59.3	8.8	5.25	148	44	347	0.48	-
41	m	72.8	6.0	4.52	152	44	182	0.19	-
109	m	78.0	3.6	4.89	158	45	220	0.83	-
130	m	81.1	9.4	4.49	126	39	307	0.39	-
134	m	69.9	6.9	5.25	155	45	292	22.38	-
140	f	79.3	11.1	5.67	167	52	393	96.85	PV
142	m	65.6	7.8	4.69	151	47	267	0.75	-
181	m	67.2	6.5	4.54	100	32	233	0.34	-

WBC indicates white blood cells; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; PLT, platelets; MA, mutant allele; MPN, myeloproliferative neoplasms; PV, polycythemia vera.
Diagnosis of PV was made according to actual WHO criteria, based on *JAK2 V617F* mutational status and elevated hemoglobin (>185 g/L in men or >165 g/L in women).

of 1.5%, ranging from 0.3% to 5.6% [28]. *JAK2 V617F* mutation frequency in PAD patients was significantly increased compared with healthy subjects from the KORA F4 study [OR = 5.35 (1.89–15.15); $P < 0.001$]. Difference between the two groups remained significant after adjustment for age and gender in multivariate regression analysis [OR = 4.40 (1.52–12.78); $P = 0.006$]. Even after exclusion of the patient with PV, association between the *JAK2 V617F* mutation and PAD remained significant [adjusted OR = 4.00 (1.34–11.92); $P = 0.013$].

Discussion

To the best of our knowledge, this study is the first to examine *JAK2 V617F* mutational status in PAD patients. Here, we identified nine PAD patients positive for the *JAK2 V617F* mutation (3.1%). Among these, one patient showed elevated hemoglobin values indicating PV.

Presence of the *JAK2 V617F* mutation has been closely linked to MPN [15–20] and included in the WHO classification and diagnostic algorithms for these diseases [21]. The fact that the *JAK2 V617F* mutation is more common than the anticipated number of MPN has also been realized by other studies, including subjects without overt signs of MPN [22–27]. It has been suggested, that the *JAK2 V617F* mutation per se is probably neither sufficient to induce a MPN nor associated with disease progression, but may represent an early molecular event in the development of blood disorders [24,27].

It should be noted, that most of our *JAK2 V617F* positive patients without overt signs of MPN showed a very low mutant allele burden compared with the patient with PV. Therefore, a sufficient allele load of *JAK2 V617F* appears necessary for the manifestation of MPN. However, in *JAK2 V617F* positive patients with ET, mutant allele burden has been reported to start from 1% [35,36] indicating that allele burden at a low level may be sufficient to induce MPN. In contrast, one of our patients (n° 134) showed a mutant allele load over 20%, but no hematologic signs of MPN. Thus, besides the *JAK2 V617F* mutation, additional genetic or environmental factors appear to be necessary for the progression into an overt MPN phenotype.

Observed *JAK2 V617F* mutation frequency in PAD patients was significantly increased compared with mutation frequency of healthy subjects without a medical history of vascular disease and may be potentially involved in the manifestation of the disease. Indeed, the JAK/STAT pathway is a critical regulator of inflammatory processes in various cells and is involved in the production and signal transduction of several pro-inflammatory cytokines, such as interleukin-6 (IL-6) [12,37–39]. IL-6 acts in an autocrine, paracrine as well endocrine manner and contributes to various atherogenic processes such as proliferation of vascular smooth muscle cells [40], B-cell differentiation [41], T-cell activation [41] and the induction of several acute phase proteins, including C-reactive protein (CRP) [4,42]. Although its function is not fully defined, it is probable that CRP itself plays a significant role in the progression of atherosclerosis [4] and predicts future risk of developing PAD [43]. The *JAK2 V617F* mutation results in a constitutive activation of the JAK2/STAT signalling pathway [15–17] and may enhance cytokine signalling, resulting, e.g., in an increased synthesis of CRP. Indeed, *JAK2 V617F* allele burden has been significantly associated with increased CRP levels in patients with ET or PV [44]. Therefore, one can speculate that the *JAK2 V617F* mutation contributes to a pro-atherogenic phenotype, which is supported by the link between inflammation, JAK/STAT pathway and atherosclerosis [12,14].

However, *JAK2 V617F* mutant load is low in most of our patients and CRP values did not differ significantly between PAD patients with or without the *JAK2 V617F* mutation. Thus, it can be assumed that most subjects included in our study did not reach the threshold value of *JAK2 V617F* allele burden, necessary to affect systemic inflammation. Indeed, only a *JAK2 V617F* allele burden greater than 50% has been significantly correlated with increased CRP levels in patients with MPN [44]. Therefore, due to the observed low allele burden in most of our *JAK2 V617F* positive patients, a causal effect of the *JAK2 V617F* mutation on inflammation and consequently on the development of PAD remains questionable.

Vice versa, it appears more likely that factors promoting atherogenesis also promote *JAK2 V617F* mutagenesis and that the increased prevalence of the *JAK2 V617F* mutation in PAD patients is caused by risk factors of the disease. Smoking, e.g., is one of the strongest risk factors of PAD [8] and is further associated with an increased rate of mutagenesis and cancer [29,30,45]. Indeed, in regard to the *JAK2 V617F* mutation, a recent study on hospitalized smokers and nonsmokers found the mutation in higher frequencies among smokers than nonsmokers [26]. The authors suggested that accelerated erythropoiesis in smokers lead to more DNA repair errors and thus to an increased susceptibility to the *JAK2 V617F* mutation. Smoking, therefore, may contribute to increased *JAK2 V617F* mutagenesis and the high smoking rate within our PAD patients may have contributed to the observed accumulation of the *JAK2 V617F* mutation in these patients.

Furthermore, smoking as well as other PAD risk factors including T2DM, obesity, and dyslipidemia are related to chronic inflammation [46–48] and PAD patients are characterized by a low-grade systemic chronic inflammatory state [3,4]. Chronic inflammation is associated with an increase in cytokines and oxidative stress, promoting epigenetic changes, and mutagenesis [13,49–51]. Consequently, chronic inflammation has been strongly linked with the development of several cancers, including certain hematologic neoplasms [13,49–52].

However, chronic inflammation as a potential initiating event and driver of clonal evolution in MPN has been barely studied [13]. Notably, a link between chronic inflammation, cytokines, and clonal evolution has been recently demonstrated by the finding of Fleischman et al. [53] that tumor necrosis factor-alpha facilitates clonal expansion of *JAK2 V617F* positive cells in MPNs. Therefore, it may be assumed that, compared with healthy people, PAD patients are more susceptible to the *JAK2 V617F* mutation due exposure to a certain state of systemic chronic inflammation, caused by PAD risk factors or the disease itself.

Interestingly, aspirin use was distinctly decreased in patients with the mutation compared with *JAK2 V617F* negative subjects. In PAD patients, aspirin is normally used as an anti-thrombotic agent. Its anti-thrombotic as well as its well known anti-inflammatory effects occur through the inhibition of cyclooxygenases [54]. Notably, numerous studies have shown that regular use of aspirin is associated with a reduced risk for colorectal, oesophageal, breast, lung, prostate, liver, and skin cancers (as recently reviewed by Alfonso et al. [55]). The precise mechanisms leading to its anticancer effects are not clearly established, although multiple mechanisms affecting enzyme activity, transcription factors, cellular signalling and mitochondrial functions have been proposed [55]. Aspirin shows anti-oxidant properties and is a direct quencher of the genotoxic hydroxyl radical, exhibiting a protective effect against oxidative stress and DNA damage [56]. Therefore, due its anti-oxidant properties, aspirin use may have protected PAD patients against *JAK2 V617F* mutagenesis. This hypothesizes is in line with our second assumption that increased *JAK2 V617F* mutation incidence in PAD patients is due to increased exposure to a certain state of systemic chronic inflammation and oxidative stress.

Our study has several limitations: First, it should be noted, that number of *JAK2 V617F* positive patients is small and further studies are warranted to confirm our observations, particularly the close association between aspirin use and lowered prevalence of the *JAK2 V671F* mutation in PAD patients. Also, we cannot exclude that some associations may have reached statistical significance within a larger population. Nethertheless, our study represents the first study concerning the *JAK2 V617F* mutation in PAD patients so far. Our PAD population is well characterized and meticulously monitored. Provided data, therefore, allow clinical interpretations regarding the *JAK2 V617F* mutational status in PAD patients and represent a valuable basis for subsequent studies. Furthermore, characteristics of control subjects are limited to age and gender. A more detailed comparison between characteristics of PAD patients and control subjects may have pointed to individual risk factors leading to increased *JAK2 V617F* prevalence in PAD patients. Thus, our observations remain descriptive and the underlying mechanisms remain to be elucidated.

We conclude that the prevalence of the *JAK2 V617F* mutation is increased in PAD patients compared with healthy subjects, although the absolute frequency of the mutation is generally low. It appears likely that the mutation is induced by risk factors of PAD causing chronic inflammation and oxidative stress. This assumption is supported by our observation that the frequency of the mutation in PAD patients is modulated by the anti-inflammatory drug aspirin. Therefore, we hypothesize that the *JAK2 V617F* mutation is not a risk factor for PAD but vice versa the mutation may be induced by the pathophysiological conditions prevalent in PAD patients. Future studies are warranted to confirm our observations and to define the underlying mechanisms behind our findings.

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