

research article

Carotid artery stiffness, digital endothelial function, and coronary calcium in patients with essential thrombocytosis, free of overt atherosclerotic disease

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Background. Patients with myeloproliferative neoplasms (MPNs) are at increased risk for atherothrombotic events. Our aim was to determine if patients with essential thrombocytosis (ET), a subtype of MPNs, free of symptomatic atherosclerosis, have greater carotid artery stiffness, worse endothelial function, greater coronary calcium and carotid plaque burden than control subjects.

Patients and methods. 40 ET patients without overt vascular disease, and 42 apparently healthy, age and sex-matched control subjects with comparable classical risk factors for atherosclerosis and Framingham risk of coronary disease were enrolled. All subjects were examined by physical and laboratory testing, carotid echo-tracking ultrasound, digital EndoPat pletysmography and CT coronary calcium scoring.

Results. No significant differences were found between ET patients and controls in carotid plaque score [1 (0-1.25) vs. 0 (0-2), $p=0.30$], β -index of carotid stiffness [7.75 (2.33) vs. 8.44 (2.81), $p=0.23$], pulse wave velocity [6.21 (1.00) vs. 6.45 (1.04) m/s; $p=0.46$], digital reactive hyperemia index [2.10 (0.57) vs. 2.35 (0.62), $p=0.07$], or augmentation index [19 (3-30) vs. 13 (5-22) %, $p=0.38$]. Overall coronary calcium burden did not differ between groups [Agatston score 0.1 (0-16.85) vs. 0 (0-8.55), $p=0.26$]. However, significantly more ET patients had an elevated coronary calcium score of >160 [6/40 vs. 0/42, $p < 0.01$].

Conclusions. No significant differences between groups were found in carotid artery morphology and function, digital endothelial function or overall coronary calcium score. Significantly more ET patients had an elevated coronary calcium score of >160 , indicating high cardiovascular risk, not predicted by the Framingham equation.

Key words: arterial wall; functional properties; morphological properties; calcium score; Framingham risk score; myeloproliferative disease

Introduction

Philadelphia chromosome-negative chronic myeloproliferative neoplasms (MPNs) are clonal haematopoietic stem cell disorders, traditionally

divided into essential thrombocytosis (ET), polycythemia vera (PV), and primary myelofibrosis (PMF). Transitions between these disease entities are common, and may represent a continuum from early disease to the advanced myelofibrosis

stage and finally to leukemic transformation.¹ The Janus kinase JAK2 V617F mutation is detected in more than 95% of patients with PV and in approximately 50% of patients with ET and PMF.² It is not known in detail, how the Janus kinase (JAK) signal pathway dysregulation affects MPNs initiation and evolution, but elevated biomarkers of chronic inflammation have been described in all MPNs entities.³⁻⁹ In addition to thrombotic and haemorrhagic complications and bone marrow failure in the advanced stage, patients with MPNs often suffer atherothrombotic events.⁹⁻¹¹ Thrombosis may involve the veins as well as the arteries, with acute coronary disease being the most prevalent manifestation of the latter.¹⁰ Over a period of 10 years, about 10% of patients with PV and ET suffered myocardial infarction.¹¹

Chronic inflammation plays an important role in the development of atherosclerosis in general population.^{12,13} In MPNs, it seems that chronic inflammation could be a trigger and promoter of the clonal expansion of leukocytes and platelets which release inflammatory cytokines.¹⁴ Since inflammatory cytokines contribute to leukocyte and platelet generation, a positive feedback loop is established¹⁴, contributing to premature atherosclerosis in patients with MPNs.^{8,9,15}

Ultrasonographic measurement of carotid arterial stiffness has been proposed as a sensitive method for detecting early vascular changes.¹⁶⁻¹⁸ Endothelial dysfunction is associated with cardiovascular risk¹⁹, and also a contributor to the progression of atherosclerosis.²⁰ Coronary artery calcium scanning is the most reliable predictor of coronary events in subjects with intermediate cardiovascular risk.²¹ Little is known about functional and morphological properties of the arterial wall and the prevalence of asymptomatic coronary atherosclerosis in patients with JAK2 V617F positive myeloproliferative disease.

Our aim was to test whether patients with JAK2 V617F positive MPNs, without clinically manifest atherosclerosis, have more prevalent asymptomatic carotid plaques, greater carotid artery stiffness, greater coronary calcium burden and worse digital endothelial function than apparently healthy control subjects, matched for classical risk factors for atherosclerosis.

Patients and methods

Patients were recruited from University Medical Centre Ljubljana, Department of Haematology

among JAK2 V617F positive patients with ET, treated between 2011 and 2014. Among 180 ET consecutive patients, 124 were JAK2 positive and 61 did not have a personal history of clinically manifest atherosclerotic vascular disease (angina pectoris, myocardial infarction, transient ischemic attack, ischemic stroke, peripheral arterial disease or known aortic disease). Among those, 40 patients gave their informed consent for participating in the cross-sectional study of functional and morphological properties of the carotid arteries, coronary calcium burden and endothelial function of the digital arteries. The control group was recruited among apparently healthy employees of the University Medical Centre Ljubljana and their relatives, aiming to match the patient group regarding age and sex. After screening 57 volunteers, 42 apparently healthy subjects were selected matching the ET patients for age, sex distribution and classical risk factors for atherosclerosis.

All participating subjects had to be at least 18 years old, not pregnant, and free of documented or clinically suspected atherosclerotic vascular disease. After giving their informed consent, all subjects were questioned for their medical history according to a structured questionnaire, examined physically and drawn blood for laboratory testing. Subsequently, their extracranial carotid arteries were examined by ultrasound, reactive hyperemia response of the digital arteries was assessed by EndoPat pletysmography, and coronary calcium burden was assessed by CT. Workup of each study participant was done in single visit, between January 2014 and August 2015, strictly on Fridays between 12.00 and 16.00 hours, in the facilities of the Clinical Department for Vascular Diseases and Department of Nuclear Medicine, University Medical Centre Ljubljana. The study was approved by the Committee for Medical Ethics of the Republic of Slovenia with the decision letter 154/05/12.

The 10-year risks of coronary heart disease, myocardial infarction, stroke and overall cardiovascular disease were calculated according to the Framingham risk equations, taking into account the subjects age, sex, smoking status, presence of diabetes, systolic blood pressure total serum cholesterol and HDL-cholesterol.²² Left ventricular hypertrophy as determined by EKG was not taken into account. The Framingham risk calculator in Microsoft Excel was used for the calculations.²³

The extracranial carotid arteries (common, internal and external carotid on both sides) were examined by a single ultrasonographer, using an Aloka

prosound $\alpha 7$ (Hitachi Aloka Medical, Ltd., Japan) machine with a linear vascular probe (working frequency of 5-13 MHz). Testing was performed with subjects comfortably lying supine in a quiet room with the air temperature of 22-24°C.

Asymptomatic carotid atherosclerosis was assessed by identifying carotid plaques, which were defined as focal lesions exceeding the intima-media thickness by at least 50% or reaching an absolute thickness of at least 1.5 mm in two orthogonal projections. Scoring of atherosclerotic plaques was performed by a modification of the methodology used in the Rotterdam Study.²⁴ The extracranial carotid arteries were divided into three sectors on each side: the common carotid artery and its bulb, the internal carotid artery, and the external carotid artery. At least one plaque in any sector was scored 1 point, while the absence of plaques was scored as 0. Thus, the carotid plaque score ranged from 0 (absence of plaques) to 6 (plaques in all sectors).²⁴

Echo-tracking of the common carotid arterial wall 2 cm proximal to the bulb was used to the β -stiffness index (β)²⁵ and to estimate pulse wave velocity (PWV)²⁶. The β -stiffness index was calculated as:

$$\beta = \ln(P_{\max} / P_{\min}) / [(D_{\max} - D_{\min}) / D_{\min}]$$

where P_{\max} was the systolic blood pressure, P_{\min} the diastolic pressure; D_{\max} the maximum arterial diameter and D_{\min} the minimal arterial diameter.

The PWV was estimated according to the formula:²¹

$$PWV = \sqrt{((\beta \times P_{\min}) / 2q)}$$

where q was the specific mass of blood ($q = 1050 \text{ kg/m}^3$).

Coronary artery calcium scanning was performed on a Biograph M 128-row PET-CT scanner (Siemens, Erlangen, Germany). We used a non-contrast protocol with sequential, prospective ECG triggering, rotation time 0.33 sec, tube voltage 120 kV, CARE Dose 4D, slice thickness 3 mm, with no slice overlap. Scanning was done in sustained breath hold, from the carina to the base of the heart. Post-processing was done on the Syngo Leonardo workstation. Evaluation of the dataset of every study subject was done three times, and the calculated average value was used for further analysis. The coronary calcium burden was expressed as the Agatston score.²⁷

Endothelial function of the digital arteries was measured by digital pletysmography before and after a 5-min arterial occlusion of the forearm by inflating a cuff to 60 mmHg more than the arterial blood pressure in order to assess the response to reactive hyperemia by the apparatus EndoPAT2000 (Itamar Medical REF, Israel). All subjects were examined in the fasting state and were requested to abstain from drinking coffee or smoking at least 3 hours before the examination, and to abstain from drinking alcohol at least 10 hours before the examination. Testing was performed with subjects comfortably lying supine in a quiet room with the air temperature 22-24°C.

The reactive hyperemia index (RHI) and augmentation index (AI) were determined.²⁸ RHI was calculated by the formula:

$$RHI = (A/B) / (C/D)$$

where A is the post-occlusion pulse wave amplitude (PWA) of the occluded hand, B the baseline PWA of the occluded hand, C the post-occlusion PWA of the contralateral hand, and D the baseline PWA of the contralateral hand.

The AI was determined from the shape of the arterial pulse wave by the EndoPAT 2000 software which distinguished between the primary pulse wave (P1) and the reflected pulse wave (P2) by the formula: $AI = ((P2-P1)/P1) \times 100$.²⁸ The results were normalized to a heart rate of 75/min.

All sets of data were tested for normality of distribution using the normal-quintile plot, calculating the correlation coefficient and checking it for the critical value that would warrant rejection of normal distribution with an α -error probability of 0.05. Normally distributed data are presented as mean and standard deviation, while non-normally distributed data are presented as median and range between the 1st and 3rd quartile. Differences between subjects with ET and control subjects were tested by the chi-square test for discrete variables, for normally distributed continuous variables by the paired Student's t-test for independent samples, and for non-normally distributed continuous variables by the Mann-Whitney test for independent samples.

The Pearson correlation coefficient was calculated between the Framingham prediction of coronary heart disease and the coronary calcium score, and between the coronary calcium score and the carotid plaque score of the two groups. The calculations were done by Microsoft Excel software or by the Social Science Statistics Calculators available at www.socscistatistics.com.

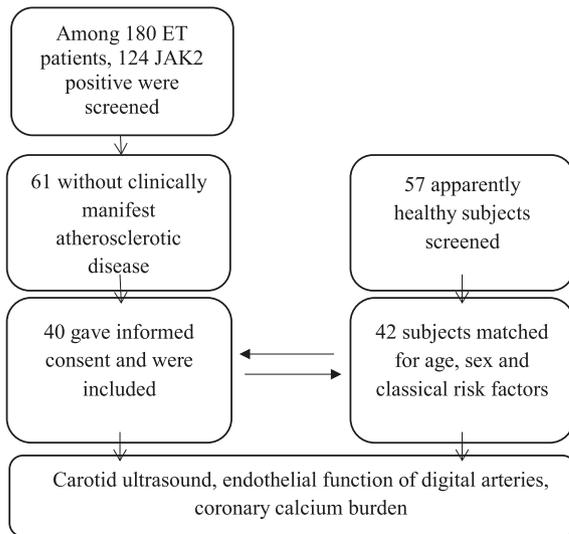


FIGURE 1. Recruitment of essential thrombocytosis (ET) and control subjects for the cross-sectional study of endothelial function and preclinical atherosclerosis.

Results

The flow chart of recruitment is shown in Figure 1

The baseline characteristics of our subjects are shown in Table 1. The groups were matched for age and sex distribution and there were no significant differences in blood pressure, blood lipids, smoking status or diabetes. Thus, there were no differences in the Framingham prediction of cardiovascular risk between the two groups (Table 2).

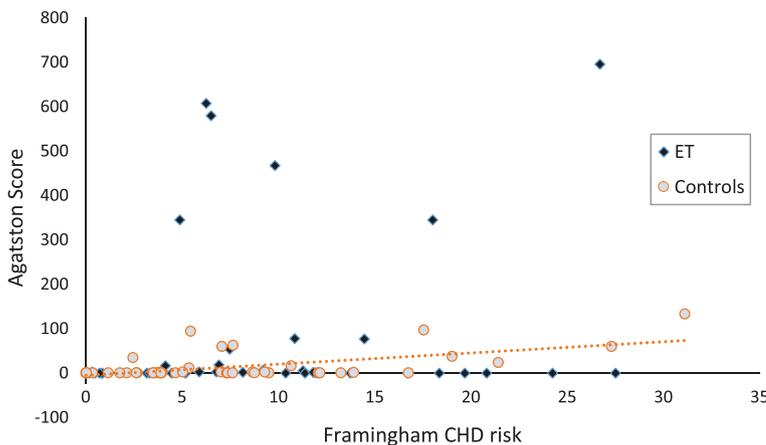


FIGURE 2. Correlation of the Framingham coronary heart disease (CHD) risk and coronary calcification (Agatston score). While a significant Pearson correlation between the Framingham CHD risk and the Agatston score was found for control subjects ($r = 0.577$, $p < 0.001$), no significant correlation was found for the patients with essential thrombocytosis (ET).

The patients with ET differed from the control group in most parameters of blood cell count, most notably the number of platelets (Table 3). No differences were found between the two groups in the carotid plaque score, carotid artery stiffness or the estimated pulse wave velocity (Table 4).

The RHI of the digital arteries showed a trend towards toward better endothelial reactivity in control subjects [2.35 (0.62) vs. 2.10 (0.57)], but the difference did not reach statistical significance ($p = 0.07$). There were also no differences in the AI, an estimate of stiffness of the conductive arteries of the upper limb (Table 5).

The majority of our patients and control subjects had their coronary arteries free of calcification (Figure 2), and the overall Agatston calcium score did not differ between the two groups. However, a significant number of patients with ET had a high calcium score not predicted by the Framingham risk equation for coronary disease (Table 6, Figure 2). While a significant correlation between the Framingham CHD risk and the Agatston was found for control subjects ($r = 0.577$, $p < 0.001$), no significant correlation was found for the patients with ET ($r = 0.197$, $p = 0.223$).

A weak correlation between the carotid plaque score and the Agatston coronary artery calcium score was found in patients with ET ($r = 0.418$, $p < 0.01$), but there was no correlation in the control group ($r = 0.063$, $p = 0.69$).

Discussion

This cross-sectional study of patients with JAK2 V617F positive ET did not find differences in carotid artery plaque score, carotid artery stiffness, endothelial function or overall coronary calcium score in comparison to control subjects, but there were more patients with a high coronary calcium score among the patients. The Framingham coronary disease risk prediction correlated with the coronary calcium score in control subjects, but not in patients with ET, indicating that Philadelphia chromosome-negative MPNs, specifically ET, represent a non-classical risk factor for coronary atherosclerosis.

Why was high calcium scoring the only parameter that differed between patients with ET and apparently healthy control subjects, whereas arterial stiffness and endothelial function did not show any significant difference?

Coronary calcium scanning is the most reliable predictor of coronary events in asymptomatic individuals with an intermediate risk according to

TABLE 1. Baseline characteristics. Numbers of subjects are given for discrete data. Mean and standard deviation are shown for normally distributed continuous data; median and interquartile range are given for non-normally distributed continuous data. Comparisons between groups were tested by χ -square¹, Student's t-test², or Mann-Whitney test³

Variable	ET patients (n= 40)	Control group (n= 42)	Comparison between groups (p)
² Age (years)	57.1 (14.1)	58.2 (13.1)	0.71
¹ Sex (M/F)	14/26	16/26	0.77
² BMI (kg/m ²)	25.4 (3.5)	27.0 (4.5)	0.07
² Waist circumference (cm)			
M	95.4 (10.4)	101.1 (10.2)	0.14
F	89.7 (8.8)	89.4 (13.6)	0.92
³ Systolic blood pressure (mmHg)	139 (129-148)	136 (130-143)	0.33
³ Diastolic blood pressure (mmHg)	80 (73-89)	80 (74-87)	0.92
² Total cholesterol (mmol/l)	5.01 (1.07)	5.23 (0.83)	0.30
² LDL-cholesterol (mmol/l)	2.73 (0.77)	2.86 (0.69)	0.42
² HDL-cholesterol (mmol/l)	1.44 (0.48)	1.63 (0.48)	0.07
² Triglycerides (mmol/l)	1.82 (0.79)	1.65 (0.82)	0.33
¹ Current smoking (yes/no)	5/35	3/39	0.41
¹ Ever smoking (yes/no)	16/24	11/31	0.12
¹ Diabetes (yes/no)	3/37	0/42	0.07
³ Serum glucose (mmol/l)	5.4 (4.7-6.1)	5.1 (4.8-5.6)	0.64
Kidney disease (yes/no)	0/40	0/42	-
² Serum creatinine (μ mol/l)	75.7 (15.0)	75.7 (14.1)	0.99
Family history of premature CVD (yes/no)	0/40	0/42	-
¹ Family history of CVD (yes/no)	16/24	16/26	0.86

BMI = body mass index; CVD = cardiovascular disease; ET = essential thrombocytosis; F = female; HDL = high density lipoprotein; LDL = low density lipoprotein; M = male

TABLE 2. Cardiovascular 10-year risk estimation by the Framingham risk equations. Median and interquartile range are shown. Comparisons between groups were tested by the Mann-Whitney test

Framingham 10-year risk calculation (%)	ET patients (n= 40)	Control group (n= 42)	Comparison between groups (p)
CHD	7.80 (3.98-13.73)	7.20 (3.57-11.37)	0.52
MI	2.87 (1.25-6.72)	2.17 (0.77-4.73)	0.47
Stroke	2.93 (1.19-5.37)	2.59 (1.49-4.09)	0.73
CVD	14.56 (7.16 – 23.68)	12.99 (6.43-19.52)	0.84

CHD = coronary heart disease; CVD = overall cardiovascular disease; ET = essential thrombocytosis; MI = myocardial infarction; Stroke = ischemic stroke

the Framingham score.²¹ Many studies have demonstrated its prognostic superiority over risk-factor based predictions, and the radiation exposure is no greater than that of mammography.²¹ Individuals with an Agatston score of >160 have a significantly increased risk for a major adverse cardiac event²⁹, and our cross-sectional study identified 6 such patients among the 40 patients with ET, but

none among the 42 control subjects. Since there was no correlation between the Framingham risk prediction and the coronary calcium score among patients with ET, this speaks for JAK2 positive ET being a non-classical risk factor for coronary atherosclerosis. With the increasing availability of coronary artery calcium scanning and its decreasing radiation exposure it might be desirable to test

TABLE 3. Blood cell count and C-reactive protein (CRP). Mixed cells denote a composite reading for monocytes, eosinophils and basophils. When CRP was reported as < 5 mg/L, a value of 2.5 mg/L was ascribed to the subject, therefore the CRP values are only an approximation. Mean and standard deviation are shown for normally distributed data; median and interquartile range are given for non-normally distributed data. The comparisons between groups were tested by Student's t-test¹, or by Mann-Whitney test²

Variable	ET patients (n= 40)	Control group (n= 42)	Comparison between groups (p)
¹ Red blood cells [10 ¹² /L]	4.37 (0.67)	4.76 (0.41)	<0.01
¹ Platelets [10 ⁹ /L]	509 (182)	243 (53)	<0.001
¹ Leukocytes [10 ⁹ /L]	7.60 (3.00)	7.02 (1.63)	0.28
¹ Lymphocytes [10 ⁹ /L]	1.67 (0.80)	2.23 (0.75)	<0.01
¹ Neutrophils [10 ⁹ /L]	5.15 (2.48)	4.15 (1.20)	0.02
² Mixed cells [10 ⁹ /L]	0.6 (0.4-0.9)	0.6 (0.5-0.7)	0.76
² CRP [mg/L]	5.0 (2.5-8.4)	5.4 (2.5-6.1)	0.27

ET = essential thrombocytosis

TABLE 4. Asymptomatic carotid plaques, carotid β -stiffness index and estimated pulse wave velocity. Mean and standard deviation are given for normally distributed continuous data, median and interquartile range are given for non-normally distributed continuous data and the number of subjects with a carotid plaque score of ≥ 2 is given. Comparisons between groups were tested by: χ -square¹, Student's t-test², or the Mann-Whitney test³

	ET patients (n= 40)	Control group (n= 42)	Comparison between groups (p)
³ Carotid plaque score	1 (0-1.25)	0 (0-2)	0.30
¹ Carotid plaque score ≥ 2 (yes/no)	10/30	14/28	0.41
² β -stiffness index	7.75 (2.34)	8.44 (2.81)	0.23
² pulse wave velocity (m/s)	6.21 (1.00)	6.45 (1.04)	0.46

ET = essential thrombocytosis

TABLE 5. Endothelial function of the digital arteries - reactive hyperemia index (RHI) and estimate of vascular stiffness - augmentation index (AI). Means and standard deviations are given for the normally distributed RHI, medians and interquartile range are given for non-normally distributed AI. Comparisons between groups were tested by the Student's t-test¹, or the Mann-Whitney test²

	ET patients (n= 40)	Control group (n= 42)	Comparison between groups (p)
¹ RHI	2.10 (0.57)	2.35 (0.62)	0.07
² AI [%]	19 (3-30)	13 (5-22)	0.38

ET = essential thrombocytosis

TABLE 6. Coronary calcium burden. Median and interquartile range are given for the Agatston score of coronary calcification, and the number of subjects with an Agatston score of > 160 is given. The comparison between groups were tested by χ -square¹- or Mann-Whitney test²

Coronary calcium burden	ET patients (n= 40)	Control group (n= 42)	Comparison between groups (p)
² Agatston score	0.1 (0-16.85)	0 (0-8.55)	0.26
¹ Agatston score > 160 (yes/no)	6/34	0/42	<0.01

ET = essential thrombocytosis

all middle aged patients with JAK2 positive MPNs for coronary calcium regardless of their perceived Framingham risk.

Carotid plaque score and the Agatston coronary artery calcium score, two markers of advanced atherosclerosis, were weakly correlated in patients with ET ($r = 0.418$, $p < 0.01$), but not at all in the control group.

Functional methods for assessing arterial stiffness and endothelial function are less robust than coronary calcium scanning and morphological carotid ultrasound examination. Although arterial stiffness and endothelial dysfunction strongly correlate with vascular risk factors and atherothrombotic events, there is no universally accepted method of their measurement and their clinical utility is not well established.^{16,19} Since arterial stiffness and endothelial function can be measured in many different ways, each research group has to focus on methods that are available to them and with which they are familiar. We chose the ultrasound based echo-tracking method to determine the local stiffness of the common carotid artery, expressed by the β -stiffness index, which has the advantage of being independent of blood pressure in a wide physiological range.²⁵ Detecting and analyzing carotid wall motion as a function of cardiac cycle by echo-tracking is straightforward, but carotid stiffness tells little about the coronary arteries, which have much greater stiffness than the common carotid arteries.³⁰ Carotid stiffness predicted cardiovascular events in patients with advanced renal disease³¹ and following renal transplantation³², but was not predictive in a broader sample of patients with manifest cardiovascular disease.³³

The most widely used non-invasive method of measuring endothelial function is flow-mediated vasodilatation of the brachial artery, which however is time-consuming and may be operator dependent.¹⁹ We used finger pletysmography/pulse amplitude tonometry with the EndoPat method which has the advantage of being relatively rapid and operator-independent.^{19,34} Endothelial dysfunction, assessed by this method correlated with traditional and metabolic cardiovascular risk factors in the third generation of the Framingham cohort.³⁵ The hyperaemic pulse amplitude response was somewhat blunted by increasing body mass index.³⁵ In our subjects, there was a trend toward lower body mass index in patients with ET in comparison to control subjects [25.4 (SD3.5) vs 27.0 (SD4.5) kg/m², $p = 0.07$]. Nevertheless, we noted a trend towards better reactive hyperaemia index in the control subjects [2.35 (SD 0.62) vs. 2.10 (SD 0.57), $p = 0.07$].

The main limitation of our study is its cross-sectional design. Each participant was examined only once, so we could not estimate the progression of atherosclerotic disease or follow the clinical outcomes. The relatively small number of patients is another important limitation, but we have recruited all actively treated patients with ET registered at our Department of Hematology, and similar studies are expected to face the same problem, since ET has a relatively low prevalence.

Also, due to a limited pool of control subjects, they were not perfectly matched to the ET patients in terms of classical risk factors for atherosclerosis, since there was a trend toward higher prevalence of diabetes, lower HDL-cholesterol and higher prevalence of ever smoking among patients with ET. However, the striking discrepancies between the Framingham risk prediction and high coronary calcium score strongly argue against classical risk factors being predominantly responsible for the advanced coronary atherosclerosis in patients with ET.

Sensitive markers of inflammation were not measured and could therefore not be correlated with endothelial function, arterial stiffness and preclinical atherosclerosis. However, the association of JAK2 positive status and markers of inflammation has been firmly established^{2,4-7,9} and all our patients with ET were JAK2 positive.

The assessment of arterial stiffness and endothelial function was limited by our methods of measurement (see above). Although all subjects were examined at the same time of day under standardized conditions, the examination period ranged for more than a year and a half, so there might have been some effects of seasonal variability on endothelial function and arterial stiffness. However, this would have affected both groups equally, since the patients and the control subjects were examined in an interspersed fashion. Also, in clinical practice patients are seen year-round and it is mandatory to use tests that are robust enough not to be dependent on many confounders.

Conclusions

In our cross-sectional study, we did not find significant differences in asymptomatic carotid plaque score, carotid stiffness, digital endothelial function or overall coronary calcium score between patients with JAK2 positive ET and control subjects. However, significantly more patients with JAK2 positive ET than control subjects had a coronary

calcium Agatston score of > 160, indicating high cardiovascular risk that was not predicted by the Framingham equation. CT calcium score is a robust, widely available and simple test which might prove useful in identifying ET patients at high risk for coronary events.

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