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ESSENTIAL THROMBOCYTHAEMIA

Diagnosis, Prognostic Aspects, and the Outcome of Finnish Patients

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Academic Dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications which will be referred to in the text by their Roman numerals (I-V). Some additional unpublished results are presented.

I. Jantunen R, Juvonen E, Ikkala E, Oksanen K, Anttila P, Hormila P, Jansson SE, Kekomäki R, Ruutu T. Essential thrombocythemia at diagnosis: causes of diagnostic evaluation and presence of positive diagnostic findings. Ann Hematol 1998; 77: 101-106

II. Jantunen R, Juvonen E, Ikkala E, Oksanen K, Anttila P, Ruutu T. Development of erythrocytosis in the course of essential thrombocythemia. Ann Hematol 1999; 78: 219-222

III. Jantunen R, Juvonen E, Ikkala E, Oksanen K, Anttila P, Ruutu T. The predictive value of vascular risk factors and gender for the development of thrombotic complications in essential thrombocythemia. Ann Hematol 2001; 80: 74-78

IV. Niittyvuopio R, Juvonen E, Kekomäki R, Oksanen K, Anttila P, Ruutu T. The predictive value of megakaryocytic and erythroid colony formation and platelet function tests on the risk of thromboembolic and bleeding complications in essential thrombocythaemia. Eur J Haematol 2004; 72: 245-251

V. Niittyvuopio R, Juvonen E, Kaaja R, Oksanen K, Hallman H, Timonen T, Ruutu T. Pregnancy in essential thrombocythaemia: experience with 40 pregnancies. Eur J Haematol 2004; 73: 431-436

ABBREVIATIONS

ADP	adenosine 5'-diphosphate
ASA	acetylsalicylic acid
ATP	adenosine 5'-triphosphate
BFU-E	burst forming unit - erythroid
CFU-E	colony forming unit - erythroid
CFU-GM	colony forming unit - granulocyte-macrophage
CFU-Meg	colony-forming unit - megakaryocyte
CML	chronic myeloid leukaemia
DNA	deoxyribonucleic acid
EPO	erythropoietin
ET	essential thrombocythaemia
GM-CSF	granulocyte-macrophage colony stimulating factor
GP	glycoprotein
G6PD	glucose-6-phosphate dehydrogenase
IFN	interferon alfa
IL-1	interleukin-1
IL-3	interleukin-3
IL-6	interleukin-6
IMDM	Iscove's Modified Dulbecco's Medium
JAK2	Janus kinase 2
MF	myelofibrosis
MDS	myelodysplastic syndrome
MPD	myeloproliferative disease
^{32}P	radioactive phosphorus
PDW	platelet distribution width
PHA-LCM	phytohaemagglutinin-stimulated leukocyte conditioned medium
PRV-1	polycythaemia rubra vera - 1 gene
PV	polycythaemia vera
PVSG	Polycythemia Vera Study Group
RNA	ribonucleic acid
RT	reactive thrombocytosis
TGF β	transforming growth factor beta
TPO	thrombopoietin
TXA_2	thromboxane A_2
vWF	von Willebrand factor
WHO	World Health Organization
XCIP	X-chromosome inactivation pattern

ABSTRACT

Essential thrombocythaemia (ET) is a myeloproliferative disease (MPD) characterized by thrombocytosis, i.e. a constant elevation of platelet count. Thrombocytosis may appear in all MPDs (ET, polycythaemia vera, chronic myeloid leukaemia, and myelofibrosis) and as a reactive phenomenon in a variety of disorders. The differential diagnosis of thrombocytosis is important, because the clinical course, need of therapy, and prognosis are different in patients with MPDs and in those with reactive thrombocytosis. ET patients may remain asymptomatic for years, but serious thrombohaemorrhagic and pregnancy-related complications may occur. The appearance of complications is difficult to predict. The aims of the present study were to evaluate the diagnostic findings, clinical course, and prognostic factors of essential thrombocythaemia in Finnish patients.

The present retrospective study consists of 170 ET patients from five hospitals diagnosed and followed during the years 1980-1996. At the time of the diagnosis the median age of the patients was 52 years, one third of them being younger than 45 years. Two thirds of the patients were diagnosed by chance. About two thirds had a platelet count $\leq 1000 \times 10^9$/l. The diagnosis of ET was supported by an increased number of megakaryocytes with an abnormal morphology in a bone marrow aspirate, aggregation defects in platelet function studies, and the presence of spontaneous erythroid and/or megakaryocytic colony formation in *in vitro* cultures of haematopoietic progenitors. About 70 % of the patients had spontaneous colony formation, while about 30 % had a normal growth pattern.

At diagnosis or during the follow-up only a fifth of the patients remained asymptomatic. Approximately half had a major thrombohaemorrhagic complication. The proportion of the patients suffering from a thrombotic complication was as high as 45 %. About a fifth had major bleeding problems. Half of the patients had microvascular symptoms. Age over 60 years predicted a risk of major bleedings, but the occurrence of thrombotic complications was similar in all age groups. In multivariate analysis, male gender, smoking in female patients, the presence of any spontaneous colony formation, and the presence of spontaneous megakaryocytic colony formation in patients younger than 45 years were identified as risk factors for thrombotic complications.

Pregnant ET patients had an increased risk of complications. Forty-five per cent of the pregnancies were complicated and 38 % of them ended in stillbirth. A previous complicated pregnancy predicted problems in subsequent pregnancies. Treatment with acetylsalicylic acid alone or in combination with platelet lowering drugs improved the outcome of the pregnancy.

The present findings about risk factors in ET as well as treatment outcome in the pregnancies of ET patients should be taken into account when planning treatment strategies for Finnish patients.

INTRODUCTION

Essential thrombocythaemia (ET) is classified with polycythaemia vera (PV), chronic myeloid leukaemia (CML), and myelofibrosis (MF) as a chronic myeloproliferative disease (MPD).Thrombocytosis, i.e. an elevation of the platelet count, is a constant feature in ET. Clonal proliferation leading to enhanced platelet production may appear in all MPDs and, occasionally, also in myelodysplastic syndromes (MDS) (Kutti 1990, Schafer 2004). Reactive thrombocytosis (RT), on the other hand, includes thrombocytosis associated with acute or chronic infections or inflammations, malignancies, iron deficiency, bleedings, as well as thrombocytosis after splenectomy (Kutti 1990, Schafer 2004).

As a result of the increased use of automated blood cell counters thrombocytosis has become a common chance finding. The differential diagnosis of thrombocytosis is crucial, since the clinical course, need of therapy, and prognosis are different in patients with RT and MPDs. The clinical course of ET is characterized by serious, even fatal, thrombotic and bleeding complications, whereas patients with RT do not suffer from thrombohaemorrhagic complications (Murphy 1983, Schafer 1984, Mitus and Schafer 1990b, Pearson 1991, Randi et al. 1991, Buss et al. 1994, Griesshammer et al. 1999a). Many patients with ET may remain asymptomatic for years, and the prediction of complications is difficult.

The present thesis deals with the diagnosis and clinical course of ET and investigates predictive factors for thrombohaemorrhagic complications which may be of use in difficult treatment decisions.

REVIEW OF THE LITERATURE

ET is a chronic myeloproliferative disease characterized by clonal proliferation of megakaryocytes and a constantly elevated platelet count (Dameshek 1951, Fialkow et al. 1981). It has been previously regarded as a disease of middle-aged or elderly patients (Tobelem 1989). However, with the frequent use of automated blood cell counters ET is increasingly diagnosed in young and asymptomatic patients (Mitus et al. 1990a, McIntyre et al. 1991, Randi et al. 1999a), and some authors consider it the most frequent of all MPDs (Tobelem 1989, Kutti 1990). According to population-based epidemiologic studies, the incidence rates of ET range from six to 25 patients per one million inhabitans annually (Mesa et al 1999, Jensen et al. 2000b, Johansson et al. 2004). There is a slight female preponderance (Cortelazzo et al. 1990, Fenaux et al. 1990, Colombi et al. 1991, Randi et al. 1991, Lengfelder et al. 1998, Besses et al. 1999, Jensen et al. 2000b). The morbidity of the patients with ET is due to thrombotic and bleeding complications (Schafer 1984). Earlier studies emphasized the importance of bleeding complications (Gunz 1980, Murphy 1983, Bellucci et al. 1986), but more recent studies have revealed that the majority of the complications are thrombotic (Cortelazzo et al. 1990, Lengfelder et al. 1998, Besses et al. 1999). The increased risk of bleeding has been associated with a high platelet count (Bellucci et al. 1986, Budde et al. 1993, Budde and van Genderen 1997), whereas factors contributing to the appearance of thrombotic events are not clearly established. The concept of risk-stratified management is generally accepted in ET (Harrison 2005a).

Pathogenesis of ET

Normal megakaryopoiesis

Megakaryopoiesis is regulated by several cytokines. The most important regulator is thrombopoietin (TPO), a polypeptide of 353 amino acids (Bartley et al. 1994, Kato et al. 1995). TPO has several effects on normal megakaryopoiesis. It increases the size and number of megakaryocytes, stimulates the expression of platelet-specific markers, and stimulates endomitosis and polyploidy in megakaryocytes (Bartley et al. 1994, de Sauvage et al. 1994, Kaushansky et al. 1994). TPO is a megakaryocyte colony-stimulating factor acting in synergy with other molecules including interleukin-3 (IL-3), stem cell factor, and erythropoietin (EPO) to

stimulate megakaryocytic growth (Broudy et al. 1995). Together with EPO, TPO also promotes the growth of erytroid progenitor cells (Kaushansky et al. 1996, Broudy et al. 1997). Transforming growth factor β1 (TGF β1) is a potent enhancer of bone marrow stromal TPO expression which, in turn, stimulates the expression of TGF β receptors on megakaryocytes, thus increasing the sensitivity of megakaryocytes to suppression by TGF β itself (Sakamaki et al. 1999). *In vivo* TPO increases the number of megakaryocytes and platelets in mice by a factor of up to ten (Bartley et al. 1994, de Sauvage et al. 1994, Lok et al. 1994) compared to the normal levels. In addition, TPO increases the numbers of all types of haematopoietic progenitors (Bartley et al. 1994, Lok et al. 1994). It has been reported that TPO sensitizes platelets to the aggregatory effects of well-known agonists, such as thrombin, collagen, and adenosine diphosphate (ADP), even though it does not cause platelet aggregation in the absence of these substances (Chen et al. 1995, Usuki et al. 1997). Platelets regulate the concentration of TPO in plasma, and the megakaryocyte mass is inversely related to the levels of TPO (Kuter and Rosenberg 1995, Emmons et al. 1996).

TPO is the ligand for the cytokine receptor c-Mpl which has been shown to be expressed on megakaryocytes, their precursors, and platelets (Vigon et al. 1992). In addition, the progeny of normal human burst forming units - erythroid (BFU-E) contains Mpl receptor ribonucleic acid (RNA) (Broudy et al. 1997). The Mpl receptors bind TPO with high affinity, internalize, and degrade it (Broudy et al. 1997, Fielder et al. 1997, Li et al. 1999).

Megakaryopoiesis in thrombocytosis

Despite an increased platelet and megakaryocytic mass in ET, TPO levels have in most studies been normal or even elevated (Tahara et al. 1996, Cerrutti et al. 1997, Horikawa et al. 1997, Pitcher et al. 1997, Hou et al. 1998, Wang et al. 1998, Harrison et al. 1999b, Li J et al. 2000). TPO levels do not differentiate between primary and reactive thrombocytosis (Cerrutti et al. 1997, Harrison et al. 1997, Hou et al. 1998). It has been suggested that the interaction between TPO and TPO receptors play a crucial role in the pathogenesis of ET, and an alteration in the feedback interaction between the ligand and its receptor may result in thrombocytosis (Li J et al. 2000). The number of platelet TPO receptors in ET is significantly decreased, TPO/receptor complex formation is reduced, and a tenfold reduction in platelet c-Mpl RNA has been reported (Horikawa et al. 1997, Harrison et al. 1999b, Li J et al. 2000). In immunohistochemical studies a

markedly decreased c-Mpl expression has been reported in up to 68 % of patients with ET (Mesa et al. 2002). Normal concentrations of c-Mpl mRNA of bone marrow cells have been detected only in a few ET patients (Moliterno et al. 1998, Yoshida et al. 1998). The platelet-dependent TPO clearance has been reported to be reduced, and may only be 25 % of the normal (Li J et al. 2000). Therefore, a defect in down-regulation obviously contributes to the normal or elevated TPO levels in patients with ET. In one study there was some evidence of insufficient protein phosphorylation stimulated by TPO binding in ET platelets (Li J et al. 2000), but this has not been verified in other studies (Horikawa et al 1997).

In familial thrombocythaemia, which is a rare chronic MPD with autosomal-dominant inheritance, activating splice mutations in intron 3 of the TPO gene leading to increased TPO synthesis as well as an activating mutation of the c-Mpl gene have been detected (Kondo et al. 1998, Wiestner et al. 1998, Ghilardi et al. 1999, Ding et al. 2004). To date, no TPO or c-Mpl mutations have been reported in sporadic ET (Kiladjian et al. 1997, Harrison et al 1998b, Taksin et al. 1999, Allen et al. 2001).

Clonality

Traditionally ET has been considered to be a clonal disorder originating from a pluripotent haematopoietic stem cell. Female cells express randomly only one active X chromosome. Clonality assays utilize the polymorphism of the X chromosome by X-chromosome inactivation patterns (X-CIPs). Fialkow et al. (1981) reported that in ET, platelets, erythrocytes, and neutrophils express a single isoenzyme type of glucose-6-phosphate dehydrogenase (G6PD) in female patients heterozygous for G6PD isoenzymes. Later studies have used different deoxyribonucleic acid (DNA) methylation patterns of active and inactive X-chromosomal genes, such as phosphoglycerate kinase, hypoxanthine phosphoribosyl transferase, and the human androgen receptor genes (Anger 1990, Gale et al. 1996). The analysis of the RNA expression of iduronate-2-sulphatase, palmitoylated membrane protein p55, and G6PD, has allowed the evaluation of clonality of platelets and subpopulation of cells (Harrison et al. 1998a). The methods exploiting X-CIPs are limited, because a significant proportion (21-31 %) of healthy females have a skewed pattern of X-chromosome inactivation and skewed X-inactivation patterns occur associated with increasing age (Busque et al. 1996, Champion et al. 1997, Gale et al. 1997). Clonality assays are informative only in approximately a quarter of patients with ET (Harrison et

al. 1999a).

In recent reports the proportion of ET patients with clonal haematopoiesis has varied from 42 to 74 % (El-Kassar et al. 1997, Ferraris et al. 1999, Harrison et al. 1999a, Chiusolo et al. 2001, Shih et al. 2002). In the study of El-Kassar et al. (1997) the majority of ET patients had clonal haematopoiesis which, in some patients, was restricted only to platelets and the megakaryocytic lineage. In other studies no evidence of restrictions to the megakaryocytic lineage could be detected (Harrison et al. 1999a, Chiusolo et al. 2001). Clonal haematopoiesis has been regarded as a prognostic factor in ET (see later) (Harrison et al. 1999a, Chiusolo et al. 2001, Shih et al. 2002), but this has not been found in all studies (El-Kassar et al. 1997).

Haematopoietic progenitors in ET

The *in vitro* cultures of haematopoietic progenitors have created new insight into the pathophysiology of ET. The first haematopoietic progenitor identified with *in vitro* cultures was termed CFU-C (colony forming unit in culture) and the colonies comprised mainly neutrophils and macrophages (Ichikawa et al. 1966, Pike and Robinson 1970). Later, Guilbert and Iscove (1976) induced an improved culture assay for granulocyte–macrophage colonies (CFU-GM) in methyl cellulose partially replacing serum by selenite, transferrin, albumin, and lecithin. This assay supports both granulocyte-macrophage and erythroid colony formation. Cultures for committed erythroid progenitors (CFU-E, colony forming unit - erythroid) (Stephenson et al. 1971) and their antecedents, burst forming units - erythroid (BFU-E) (Axelrad et al. 1973), were established by supplementing culture media with EPO. The culture assay for megakaryocyte progenitors (CFU-Meg, colony forming unit - megakaryocyte) was described later (Metcalf et al. 1975, Vainchenker et al. 1979). Different semisolid media (plasma clot, methyl cellulose), culture conditions (with either plasma or serum), and sources of progenitors (bone marrow, peripheral blood) have been used. The growth of megakaryocytic progenitors has been stimulated with phytohaemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM) or with the plasma of patients with aplastic anaemia. In ET the number of CFU-Meg in bone marrow has been reported to be normal in most studies (Komatsu et al. 1986, Juvonen et al. 1987), even though increased numbers of progenitors have also been found (Kimura et al. 1987). With one exception (Croizat et al.1983) the number of circulating CFU-Meg has been reported to be increased (Hibbin et al. 1984, Grossi et al. 1987, Han et al 1987, Juvonen et al. 1987, Mazur et al. 1988,

Florenza et al. 1989, Han et al. 1989). The growth of erythroid or granulocyte-macrophage colonies has been either increased or within normal limits (Partanen et al. 1983, Eridani et al. 1984, Eridani et al. 1987, Juvonen et al. 1987).

The *in vitro* growth of erythroid or megakaryocytic colonies without the addition of EPO or megakaryocytic growth factors is called endogenous or spontaneous colony formation. This phenomenon is seen neither in normal persons nor in patients with RT but, in contrast, it has been shown to be present in a variable proportion of patients with ET and other MPDs (Partanen et al. 1983, Eridani et al. 1984, Komatsu et al. 1986, Eridani et al. 1987, Grossi et al. 1987, Han et al. 1987, Juvonen et al. 1987, Kimura et al. 1987, Hamagucchi et al. 1988, Battegay et al. 1989, Dudley et al. 1989, Florensa et al. 1989, Han et al. 1989, Abgrall et al. 1992, Turhan et al. 1992, Juvonen et al. 1993, Florensa et al. 1995, Rolovic et al. 1995). In the previous studies (as above) the proportion of ET patients with spontaneous megakaryocytic colony formation has ranged between 63 and 100 %. The percentage of ET patients with spontaneous erythroid colony formation varies widely, approximately from 30 to 100 %, with a median of 65 % (Croizant et al. 1983, Partanen et al. 1983, Eridani et al. 1984, Eridani et al. 1987, Juvonen et al. 1987, Kimura et al. 1987, Dudley et al. 1989, Florensa et al. 1989, Turhan et al. 1992, Juvonen et al. 1993, Florensa et al. 1995, Rolovic et al 1995).

The precise mechanism of spontaneous colony formation in MPD has remained obscure. Normal human megakaryocytes synthesize and secrete a number of cytokines including IL-1α, IL−1β, IL-3, IL-6, and granulocyte-macrophage colony stimulating factor (GM-CSF) (Jiang et al. 1994, Wickenhauser et al. 1995). Cytokines originating from the added plasma have been excluded as a reason for spontaneous colony growth (Kobayashi et al. 1993). The elimination of the role of accessory cells by CD34 positive cell selection leads to the disappearance of spontaneous megakaryocyte colony formation, suggesting that other cells contribute to one or more factors relevant for the proliferation of progenitors (Kobayashi et al. 1993). Several studies have shown that megakaryocyte progenitors are highly sensitive to low concentrations of various cytokines, such as TPO, IL-3, IL-6, and GM-CSF (Kobayashi et al. 1993, Zauli et al. 1994, Taksin et al. 1999, Axelrad et al. 2000, Kawasaki et al. 2001, Mi et al. 2001), but their depletion in the culture medium is unable to inhibit spontaneous colony formation (Li Y et al. 1994). Decreased sensitivity of megakaryocyte progenitors to the inhibitory effect of negative regulators of thrombopoiesis, such as TGF β1, has also been proposed (Zauli et al. 1993). Human c-Mpl proto-

oncogene is obviously important for the spontaneous megakaryocytic colony formation in ET, because soluble c-Mpl receptors and antisense oligonucleotides directed at c-mpl RNA have been reported to inhibit spontaneous colony formation (Li Y et al. 1996), but this effect could not be detected with antibodies to TPO (Taksin et al. 1999). Recently, the presence of Janus kinase 2 (JAK2) mutation (see later) has been identified in endogeneous erythroid colonies (Baxter et al. 2005), whereas a JAK2 inhibitor blocks endogenous erythroid colony growth (Ugo et al. 2004).

Cytogenetics and molecular pathogenesis

Cytogenetic abnormalities, which are non-specific, appear only in a minority of patients with ET (Sessarego et al. 1989, Bench et al. 2001, Swolin et al. 2001). In addition, cytogenetic abnormalities in general and especially abnormalities of chromosome 17p have not been linked to the natural history of the disease but rather to transformation to acute leukaemia caused by treatment with hydroxyurea (Sessarego et al. 1989, Sterkers et al. 1998, Merlat et al. 1999, Bernasconi et al. 2002). During recent years over-expression of polycythaemia rubra vera-1 gene (PRV-1) in neutrophils, described first in the patients with PV, has been found also in ET (Temerinac et al. 2000, Klippel et al. 2002, Teofili et al. 2002, Tefferi et al. 2003). The mechanism of this finding has been suggested to be a failure to downregulate PRV-1 in granulocytes emigrating from the bone marrow to blood rather than an upregulation of the PRV-1 gene in clonal haematopoiesis (Bock et al. 2003).

The recent finding of an association between an activating JAK2 mutation and bcr/abl negative MPDs has revealed new insight into the primary oncogenic event in these disorders. JAK2 is a cytoplasmic protein tyrosine kinase that mediates the signalling downstream of cytokine receptors (Yeh and Pellegrini 1999, Rane and Reddy 2002). The cytokine receptor/ligand complex results in autophosphorylation and transphosphorylation of both the receptor and receptor-associated JAK, which activates substrate molecules such as signal transducers and activators of transcription (STAT) proteins (Carter-Su and Smit 1998, Benekli et al. 2003). The JAK/STAT signal transduction is important in cellular proliferation and cell survival (Levy and Darnell 2002, Benekli et al. 2003). The activating mutation of JAK2 has been found in 23-57 % of the patients with ET, in 65-97 % of the patients with PV, and in 35-57 % of the patients with MF (Baxter et al. 2005, Jones et al. 2005, Kralovics et al. 2005, Levine et al. 2005). It has been suggested that the resulting kinase activity caused by JAK2 mutation is critical for the phenotype

of the MPD: low kinase activity would lead to ET, intermediate activity level to PV, and very high activity level to MF (Vainchenker and Constantinescu 2005). According to the presence of the JAK2 mutation, ET patients may be divided into two biologically distinct subgroups, JAK2-positive and JAK2-negative patients (Campbell and Green 2005). The presence of JAK2 mutation in ET has been suggested to promote the development of a PV phenotype (Wolanskyj et al. 2005).

Abnormalities in platelets

A great number of functional, structural, and metabolic platelet abnormalities have been reported in ET (Table 1). They present either with hypo- or hyperfunction of platelets (Schafer 1984). However, attempts to correlate these abnormalities to clinical events have resulted in controversies (Pareti et al. 1982, Barbui et al. 1983, Baker and Manoharan 1988, Sehayek et al. 1988, Wehmeier et al. 1989, Wehmeier et al. 1990, Finazzi et al. 1996, Wehmeier et al. 1997, Raszeja-Specht et al. 2001). There are several explanations for the conflicting results. Some platelet abnormalities in ET may be secondary to platelet activation *in vitro* having thus no correlation to platelet function *in vivo* (Remaley et al. 1989). ET patients may have more than one platelet defect simultaneously (Schafer 1984). Abnormalities in platelet function studies may also vary due to methodological differences (Balduini et al. 1991), progression of the disease (Baker and Manoharan 1988, Wehmeier et al. 1989) or cytoreductive therapy (Catani et al. 1991).

There are reports indicating that factors other than platelets alone also play a role in the haemostatic complications in ET. Several studies have revealed alterations of the fibrinolytic system in MPD. Elevated plasma levels of the plasminogen activator inhibitor (PAI-1) (Cancelas et al. 1994), dysbalance between elevated platelet PAI-1 activity and low platelet activity of the tissue-type plasminogen activator (Bazzan et al. 1993), and activation of fibrinolysis (Wieczorek et al. 1995) have been described. Other possible explanations include functional abnormalities of natural anticoagulants (Bucalossi et al. 1996, Harrison et al. 2002, Amitrano et al. 2003). Recently, endothelial damage and thrombin activation have been described in ET and PV. Activated neutrophils and monocytes promote coagulation by formation of platelet-leukocyte aggregates which, in turn, promote monocyte tissue factor expression and release of inflammatory cytokines, leading to endothelial activation and damage (Celi et al. 1994, Falanga et al. 2000, Jensen et al. 2001, Villmow et al. 2002, Falanga et al. 2005).

Table 1. Platelet abnormalities in essential thrombocythaemia (based on the thesis of Perry J.J. van Genderen, 1999a).
1. Platelet function *in vitro*
a. hypoaggregation with adrenaline, adenosine 5'-diphosphate (ADP), collagen, arachidonic acid, and prostaglandin peroxides (Fabris et al. 1981, Pareti et al. 1982, Schafer 1984, Sehayek et al. 1988, Wehmeier et al. 1989, Zahavi et al. 1991)
b. spontaneous platelet aggregation (Wu 1978, Cortelazzo et al. 1980, Hehlman et al. 1988)
c. circulating platelet aggregates (Wu 1978, Villmow et al. 2002)
d. increased plasma levels of platelet-derived products (Ireland et al. 1982, Wehmeier et al. 1989)
e. circulating activated platelets (Wehmeier et al. 1991b, Griesshammer et al. 1999, Bermejo et al. 2004)
2. Platelet function *in vivo*
a. prolonged bleeding time (Murphy et al. 1978, Pareti et al. 1982, Schafer 1984)
b. reduced platelet life span (Brodsky et al. 1972, Berild et al. 1987)
c. platelet activation *in vivo* (Rocca et al. 1995, Jensen et al. 2000a)
3. Platelet morphology
a. increased platelet distribution width at normal or elevated mean platelet volume (van der Lelie and von dem Borne 1986, Sehayek et al. 1988, Majer et al. 1991, Osselaer et al. 1997)
b. hypertrophy of dense tubular and open canalicular system (Zeigler et al. 1978)
c. reduced number of dense bodies containing adenine nucleotides and serotonin (Spaet et al. 1969, Russell et al. 1981, Pareti et al. 1982)
d. reduced α-granule stores of β-thromboglobulin, fibrinogen, platelet-derived growth factor, von Willebrand factor (Boughton et al. 1978, Ireland et al. 1982, Gersuk et al. 1989, Castaman et al. 1995)
4. Platelet membrane receptors
a. reduction of glycoprotein (GP) IIb/IIIa (Gugliotta et al. 1983, Mazzucatio et al. 1989, Jensen et al. 2000a)
b. reduction of GP Ib (Mazzucato et al. 1989, Jensen et al. 2000a)
c. increase of GP IV (thrombospondin) (Legrand et al. 1991, Thibert et al. 1995, Jensen et al. 2000)
d. decreased sialylation of GP Ib and IIIa (Clezardin et al. 1985)
e. lack of α-adrenergic receptors, leading to lack of aggregation with adrenaline (Kaywin et al. 1978)
f. decreased number of prostaglandin D_2 receptors, leading to increased resistance to inhibitory prostaglandins (Cooper and Ahern 1979, Cortelazzo et al. 1988)
g. absence of GP Ia-IIa, leading to lack of aggregation with collagen (Handa et al. 1995)
h. increased expression of Fc receptors (Moore et al. 1981)
i. decrease in fibrinogen binding sites (Mazzucato et al. 1989, Mistry et al. 1991)
5. Platelet metabolism
a. abnormalities in uptake and storage of serotonin (Cortelazzo et al. 1985)
b. abnormalities in arachidonate metabolism including lipoxygenase deficiency (Okuma and Uchino 1979, Russell et al. 1981, Smith and Martin 1982, Takayama et al. 1983, Zahavi et al. 1991, van Genderen et al. 1994b, Rocca et al. 1995, Tomo et al. 1997)
6. Platelet signal transduction
a. impaired receptor-response coupling in adrenaline-insensitive platelets (Kaywin et al. 1978, Swart et al. 1985a, Swart et al. 1985b)
b. defects in calcium mobilization and exchange across platelet membranes (Fujimoto et al. 1989, Ushikubi et al. 1990)

Acquired von Willebrand syndrome

A number of studies of MPD have shown a correlation between the platelet count and the loss of large von Willebrand factor (vWF) multimers in plasma (Budde et al. 1993, van Genderen et al. 1994a, van Genderen et al. 1996, Budde et al. 1997, van Genderen et al. 1997b, Michiels et al. 2001). This results in a functionally relevant defect leading to a bleeding tendency especially with high platelet counts. vWF is a key factor in haemostasis mediating the adhesion of platelets to vascular injury site and inducing platelet aggregation (Ruggeri 2001). Regulation of the size and

function of vWF is mediated by a specific vWF cleaving protease, ADAMTS13 (Furlan et al. 1996, Levy et al. 2001). Normally the cleavage site for ADAMTS13 in the vWF subunit is protected. The function of vWF declines with increasing platelet counts in MPD and also in RT (van Genderen et al. 1996, Favoloro 2000). The disappearance of large multimers has been reported to be caused by decreased survival after secretion (van Genderen et al. 1997b) and this may be due to the increased proteolysis of vWF (Tsai 1996, Levy et al. 2001). It has been suggested that in thrombocytosis the increased platelet count facilitates the interaction between the platelet surface receptor GP Ib and vWF leading to conformational changes in vWF thus allowing ADAMTS13 to reach its cleavage site.

Diagnosis of ET

Thrombocytosis (platelet count > 400 x 10^9/l) can be found both in MPDs and in several disorders as a reactive phenomenon. RT may be present in chronic inflammation, iron deficiency, malignancy, and bleeding (Kutti 1990, Mitus and Schafer 1990b, Kutti and Wadenvik 1996). RT is far more common than myeloproliferative thrombocytosis (Griesshammer et al. 1999a). In a study of 280 patients with platelet counts at least once higher than 1000 x 10^9/l, 231 (82 %) had RT, 38 (14 %) an MPD, and 11 (4%) thrombocytosis of an unknown cause (Buss et al. 1994). The differentiation between reactive and myeloproliferative thrombocytosis is essential since RT does not predispose to thrombohaemorrhagic complications (Schafer 1984, Griesshammer et al. 1999a). The diagnosis of ET requires the exclusion of RT as well as of the other MPDs, such as PV, CML, and MF (Murphy et al. 1986, Murphy et al. 1997).

The diagnostic criteria for ET of the Polycythemia Vera Study Group (PVSG) are based on the exclusion of other causes of thrombocytosis (Table 2). In recent years there have been attempts to define positive criteria and to find appropriate tools for the diagnosis of ET (Kutti and Wadenvik 1996, Michiels and Juvonen 1997).

Table 2. Diagnostic criteria for ET according to the Polycythemia Vera Study Group. All requirements should be fulfilled to establish the diagnosis.

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1. Platelet count > 600 x 10^9/l
2. Haemoglobin < 130 g/l or normal red cell mass (males <36 ml/kg, females < 32 ml/kg)
3. Stainable iron in bone marrow or failure of iron therapy (< 10 g/l rise in haemoglobin after one month of iron therapy)
4. No Philadelphia chromosome
5. Collagen fibrosis of marrow
    a. absent or
    b. < 1/3 biopsy area without both splenomegaly and leukoerythroblastic reaction
6. No known cause for reactive thrombocytosis

Bone marrow biopsies of untreated ET patients reveal an increase in the number and clustering of giant megakaryocytes with multilobulated nuclei (Thiele et al. 1988, Thiele et al. 2000, Thiele et al. 2003, Michiels 2004a, Michiels 2004b, Thiele et al. 2005). In some studies the platelet distribution width (PDW) has turned out to be useful in the differential diagnosis of thrombocytosis (Dudley et al. 1989, Osselaer et al. 1997). The DNA content of megakaryocytes in ET patients has been reported to be higher than in patients with RT, and in two-colour flow cytometry thrombocythaemic patients show higher ploidy numbers than patients with RT (Ridell et al. 1990, Jacobsson et al. 1994). Serum TPO levels and platelet c-Mpl expression seem to be less useful due to considerable overlapping between myeloproliferative and reactive thrombocytosis (Cerrutti et al. 1997). There are several reports of the over-expression of PRV-1 gene in ET (Teofili et al. 2002, Johansson et al. 2003a, Johansson et al. 2003b, Kralovics et al. 2003, Griesshammer et al. 2004), but it has been demonstrated that PRV-1 is constitutively expressed in bone marrow cells (Bock et al. 2003), and quantitative neutrophil PRV-1 mRNA does not distinguish between myeloproliferative and reactive disorders (Tefferi et al. 2003).

Due to age-related skewing, X-chromosomal clonality studies are informative only in female patients younger than 65 years thus limiting the use of these assays only to a quarter of the patients (Harrison et al. 1999a). In some studies merely 60-70 % of assessable ET patients have shown clonal haematopoiesis, while in the rest of the patients haematopoiesis has turned out to be polyclonal (El-Kassar et al. 1997, Harrison et al. 1999a, Chiusolo et al. 2001). In a recent study using novel clonality assays with five X-chromosomal genes the presence or absence of clonal haematopoiesis could be detected in 90 % of the female patients, 85 % of whom showed clonal

haematopoiesis (Liu et al. 2003). The recently found acquired mutation of JAK2 can be detected in approximately half of the ET patients (Baxter et al. 2005, Jones et al. 2005, Kralovics et al. 2005, Levine et al. 2005). Thus, in the future it may provide a significant tool in the differential diagnosis of thrombocytosis.

There are several reports of spontaneous colony formation of haematopoietic progenitors in ET. In different studies the proportions of the patients with spontaneous erythroid or megakaryocytic colony formation have ranged from 29 to 100 % and from 63 to 100 %, respectively (Partanen et al. 1983, Eridani et al. 1984, Komatsu et al. 1986, Eridani et al. 1987, Grossi et al. 1987, Han et al. 1987, Juvonen et al. 1987, Kimura et al. 1987, Hamaguchi et al. 1988, Mazur et al. 1988, Battegay et al. 1989, Dudley et al. 1989, Florensa et al. 1989, Han et al. 1989, Abgrall et al. 1992, Turhan et al. 1992, Juvonen et al. 1993, Florensa et al. 1995, Rolovic et al. 1995, Mi et al. 2001). In the largest study thus far, 77 % of the patients with ET showed spontaneous megakaryocytic and/or erythroid colony formation, whereas 23 % of the patients did not have any kind of spontaneous colony growth (Juvonen et al. 1993). Most studies suggest that spontaneous colony formation of BFU-E or CFU-Meg is not found in RT. However, in one study using a serum-free culture technique spontaneous colony formation was detected also in RT (Sawyer et al. 1994).

Several reports have shown that the majority of patients with ET have insufficient aggregation responses in platelet aggregation studies (Yamamoto et al. 1984, Tobelem et al. 1989, Wehmeier et al. 1990, Zahavi et al. 1991). Most frequently aggregation abnormalities are detected, not only in response to adrenaline, but also in response to collagen and ADP. There is, however, considerable overlapping between myeloproliferative and reactive thrombocytosis, and there is no clear evidence of a clinical relevance of platelet aggregation studies in the diagnostic evaluation of thrombocytosis (Sehayek et al. 1988, Majer et al. 1991).

The spleen volume studied by ultrasound or scintigraphy has been shown to differentiate between myeloproliferative and reactive disorders (Messinezy et al. 1988, Revesz et al. 1993, Picardi et al. 2002).

Dudley et al. (1989) have proposed a simple scoring system for the positive diagnosis of ET consisting of splenic enlargement on scan, presence of spontaneous colony formation of BFU-E, elevated PDW, elevated ATP/ADP ratio or the presence of clinical ischaemia. A proposal for new

diagnostic criteria for ET, which also considers the bone marrow findings and the presence of spontaneous colony formation, is shown in Table 3. The WHO classification of tumours published in 2001 emphasizes the histopathology of the bone marrow (Table 4). According to the WHO classification, the positive diagnostic criteria for ET are a sustained platelet count $\geq$ 600 x 10^9/l and a bone marrow biopsy showing proliferation of the megakaryocytic lineage. Other MPDs, MDS, and RT should be excluded.

A proposed updated version of the PVSG criteria for ET includes the JAK2 mutation as a main diagnostic hallmark (Table 5) (Campbell and Green 2005).

Table 3. Proposal for revised diagnostic criteria for ET (Michiels and Juvonen 1997).

Diagnostic
A1. Platelet count in excess of 400 x 10^9/l and no known cause for RT.
A2. Increase in the number and clustering of enlarged and mature megakaryocytes with hyperploid nuclei in bone marrow specimens.
A3. No preceding or other allied subtype of myeloproliferative disorder or myelodysplastic syndrome.
Confirmative
B1. Normal or elevated leukocyte alkaline phosphatase score, normal erythrocyte sedimentation rate, and no fever or infection.
B2. Normal or increased cellularity of bone marrow with or without the presence of reticulin fibers in biopsy material.
B3. Splenomegaly on palpation or diagnostic imaging.
B4. Spontaneous erythroid colony formation and/or spontaneous megakaryocyte colony formation in *in vitro* culture.

Table 4. WHO diagnostic criteria for ET (Imbert et al. 2001). All positive and negative criteria should be fulfilled to establish the diagnosis.

Positive criteria
1. Sustained platelet count > 600 x 10^9/l
2. Bone marrow biopsy specimen showing proliferation mainly of the megakaryocytic lineage with increased numbers of enlarged, mature megakaryocytes
Negative criteria
1. No evidence of PV
Normal red cell mass or Hb < 18.5 g/dl in males, 16.5 g/dl in females
Stainable iron in marrow, normal serum ferritin or normal MCV
If the former condition is not met, failure of iron trial to increase red cell mass or Hb levels to the PV range
2. No evidence of CML
No Philadelphia chromosome and no *bcr-abl* fusion gene
3. No evidence of chronic idiopathic myelofibrosis
Collagen fibrosis absent and reticulin fibrosis minimal or absent
4. No evidence of MDS
No del(5q), t(3;3)(q21;q26), inv(3)(q21;q26)
No significant granulocytic dysplasia, few if any micromegakaryocytes
5. No evidence that thrombocytosis is reactive due to:
Underlying inflammation, infection, neoplasm or prior splenectomy

Table 5. A proposed updated version of the PVSG criteria for ET (Campbell and Green 2005). Diagnosis of ET requires A1 + A2 + B3-6 (JAK2 mutation-positive ET) or A1 + B1-6 (JAK2 mutation-negative ET).

A1. Platelet count > 600 x 10^9/l
A2. Acquired JAK2 mutation
B1. No cause for a reactive thrombocytosis
- e.g., normal inflammatory indices
B2. No evidence of iron deficiency
- stainable iron in the marrow or normal red cell mean corpuscular volume
B3. No evidence of PV
- haematocrit < midpoint of normal range or normal red cell mass in presence of normal iron stores
B4. No evidence of chronic myeloid leukaemia
- no Philadelphia chromosome or bcr-abl gene rearrangement
B5. No evidence of myelofibrosis
- no collagen fibrosis and $\leq$ 2 reticulin fibrosis (using 0-4 scale)
B6. No evidence of a myelodysplastic syndrome
- no significant dysplasia
- no cytogenetic abnormalities suggestive of myelodysplasia

Clinical manifestations

At diagnosis the mean age of ET patients has been shown to be 50 - 60 years, and about a quarter of the patients are younger than 40 years (Michiels 2004b). There is a slight female preponderance (Table 6). Morbidity in ET is due to thrombotic and haemorrhagic events. The patients may remain asymptomatic for long periods, but serious, even life-threatening vascular events may occur also in young patients (Millard et al. 1990, Mitus et al. 1990a).

Thrombosis

Thrombotic complications including both arterial and venous thrombosis are the main cause of morbidity in ET (Iland et al. 1983, Bellucci et al. 1986, Grossi et al. 1988, Hehlmann et al. 1988, Lahuerta-Palacios et al. 1988, Chistolini et al. 1990, Cortelazzo et al. 1990, Fenaux et al. 1990, Colombi et al. 1991, Randi et al. 1991, Wehmeier et al. 1991a, Regev et al. 1997). Disturbances in cerebral or coronary circulation may lead to ischaemia and infarction even in young patients without any cardiovascular risk factors (Lahuerta-Palacios et al. 1988, Millard et al. 1990, McIntyre et al. 1991, Scheffer et al. 1991, Host and Saunamäki 1992, Koudstaal and Koudstaal 1997, Ravandi-Kashani and Schafer 1997, Daya et al. 2004). Thrombosis may also be located in peripheral, skin, and abdominal arterial circulation (Chistolini et al. 1990, Cortelazzo et al. 1990, Colombi et al. 1991, Griesshammer et al. 1997, Ravandi-Kashani and Schafer 1997). ET-related disturbances of the cerebral circulation include transient ischaemia attacks (TIAs), cerebral infarction, migraine, and non-focal neurological symptoms such as dysarthria, visual disturbances, and postural instability (Preston et al. 1979, Jabaily et al. 1983, Colombi et al. 1991, Michiels et al. 1993, Arboix et al. 1995, Koudstaal and Koudstaal 1997, Michiels et al. 1997, Randi et al. 1998). A characteristic feature of the peripheral circulation is erythromelalgia, manifested in painful burning sensations in warm and red extremities. This platelet-mediated arteriolar inflammation and thrombosis may lead to acrocyanosis and peripheral gangrene (Preston et al. 1974, Salem et al. 1980, Michiels et al. 1985, Michiels and ten Kate 1992, Michiels et al. 2004c). In recent studies the incidence of venous thrombotic events has been less frequent than that of arterial events (Hehlmann et al. 1988, Cortelazzo et al. 1990, Fenaux et al. 1990, Colombi et al. 1991). Deep vein thrombosis and pulmonary embolism are the most common venous complications, whereas thrombosis of portal, hepatic, splenic, sagittal, and retinal veins occurs with a lower frequency (Hehlmann et al. 1988, Cortelazzo et al. 1990, Fenaux

et al. 1990, Colombi et al. 1991, Imasawa and Iijima 2002, Randi et al. 2002).

Bleeding

ET is characterized by a tendency for recurrent bleedings from mucous membranes and easy bruising. Serious bleedings including gastrointestinal or intracranial haemorrhage are relatively infrequent and mostly associated with antiaggregation therapy (Hehlmann et al. 1988, Fenaux et al. 1990, Colombi et al. 1991). ET patients are also prone to postoperative bleedings after surgical procedures or after dental extraction (Schafer 1984, Mitus et al. 1990b).

Incidence of thrombohaemorrhagic complications

ET may be diagnosed as an incidental finding by routine blood testing. In different studies, some of which include also children, the proportion of asymptomatic patients at diagnosis varies from 11 to 73 % (Table 6). Of the symptomatic patients the majority presents with microvascular disturbances (Bellucci et al. 1986, Chistolini et al. 1990, Cortelazzo et al. 1990, Fenaux et al. 1990, Colombi et al. 1991). The incidence of thrombosis varies widely, largely depending on whether microvascular symptoms are included in the thrombotic events or not. Major thrombotic complications (excluding microvascular symptoms) at diagnosis have been reported in 9-43 % of the ET patients (Table 6), but Hehlmann et al. (1988) found an incidence as high as 80 %. After the diagnosis the reported rate of thrombosis varies from 7 to 17 % (Barbui et al. 2004b).

Bleeding complications are less frequent than thrombotic events (4-38 %) and only a minority of the bleedings are serious (Table 6). In a review of 809 ET patients from 11 retrospective studies, 36 % of the patients were asymptomatic at diagnosis, 41 % experienced microvascular symptoms, and 20 % had major arterial thrombosis, 17 % bleeding and 4 % venous thrombosis (Griesshammer et al. 1997).

Risk factors for thrombotic and bleeding complications

Several studies have shown that a previous thrombotic event indicates an increased risk of thrombosis (Chistolini et al. 1990, Cortelazzo et al. 1990, Colombi et al 1991, Besses et al. 1999). The predictive value of other risk factors for thrombosis has varied in different studies. Age and sex have been found to correlate with the risk of thrombosis in some studies (Cortelazzo et al. 1990, Randi et al. 1991, Randi et al. 1992, Besses et al. 1999, Shih et al. 2002), whereas in other studies no such correlation has been found (Chistoloni et al. 1990, Fenaux et al. 1990, Colombi et al. 1991, Watson and Key 1993, Bazzan et al. 1999). The platelet count does not predict thrombotic complications (Bellucci et al. 1986, Hehlmann et al. 1988, Chistolini et al. 1990, Cortelazzo et al. 1990, Fenaux et al. 1990, Colombi et al. 1991, Randi et al. 1991, Watson and Key 1993, Bazzan et al. 1999, Besses et al. 1999). The association of conventional cardiovascular risk factors with thrombotic complications in ET has been unclear. Two retrospective studies have assessed the predictive value of these factors as the primary goal. One study revealed smoking (Watson and Key 1993) and another hypercholesterolaemia (Besses et al. 1999) as a risk factor in ET. In addition, in the prospective study of Cortelazzo et al. (1995) which was not primarily aimed at studying risk factors, smoking correlated with an increased risk of thrombosis. However, in most studies conventional cardiovascular risk factors have not had any prognostic significance (Hehlmann et al. 1988, Cortelazzo et al. 1990, Fenaux et al. 1990, Bazzan et al. 1999). Spontaneous megakaryocytic colony formation has in one previous study been associated with an increased risk for complications (Juvonen et al. 1993), whereas spontaneous erythroid colony formation showed no predictive value in three other studies (Ciaudo et al. 1998, Chiusolo et al. 2001, Vannucchi et al. 2004). Clonal haematopoiesis (Harrison et al. 1999a, Chiusolo et al. 2001, Shih et al. 2002, Vannucchi et al. 2004) and PRV-1 mRNA expression (Johansson et al. 2003b, Griesshammer et al. 2004) have been associated with an increased risk for thromboembolic events.

The frequency of bleeding complications increases with the acquired von Willebrand syndrome, when the platelet count rises above 1000 x 10^9/l (Bellucci et al. 1986, Fenaux et al. 1990, van Genderen et al. 1994a, Tefferi et al. 2001b). Bleeding complications are also associated with anti-aggregation therapy (van Genderen et al. 1997a, Schafer 2004).

Table 6. Characteristics and incidence of thrombotic and bleeding complications of patients with ET at diagnosis. Age and platelet counts are expressed as means (range).

Author	No. of patients	Age	Sex ratio (F:M)	Platelet count (x 10^9/l)	Thrombosis (%)	Bleeding (%)	Microvascular symptoms (%)	Asymptomatic (%)
Bellucci et al. (1986)	94	50 (6-90)	1.76	1200	22	38	42	67
Hehlmann et al. (1988)	61	58 (10-82)	1.10	897 (300-4000)	84[1]	13	NA	11
Chistolini et al. (1990)	100	49 (2-80)	1.27	1140 (1000-3750)	9	10	29	52
Cortelazzo et al. (1990)	100	50 (17-82)	1.56	1135 (600-3000)	20	10	36	34
Fenaux et al. (1990)	147	60 (18-83)	1.45	NA (700-2900)	18	18	34	36
Colombi et al. (1991)	103	59 (9-88)	1.34	NA	24	4	33	73
McIntyre et al. (1991)	56	29 (12-40)	1.40	NA	21	7	NA	70
Randi et al. (1991)	97	54 (40-73)	1.43	1090	45[1]	38	NA	28
Lengfelder et al. (1996)	101	58 (10-83)	1.19	844 (609-4000)	43	13	43	37
van Genderen et al. (1997a)	68	57	0.70	922	60[1]	7	9	21
Besses et al. (1999)	148	61 (11-85)	1.79	898 (600-4006)	25	9	29	57
Jensen et al. (2000a)	96	67 (18-87)	2.60	1102	14	9	23	52

NA = not available
[1] Includes also microvascular symptoms

Treatment

The need for therapy and the optimal treatment of ET has been an area of debate. The platelet count does not predict the risk of complications. Many patients are asymptomatic at diagnosis and may remain asymptomatic for long periods. However, serious, even life-threatening complications may occur also in young patients, and an individual patient may suffer from both thrombotic and bleeding events at the same time. On the other hand, the potential risk of inducing secondary leukaemia with platelet lowering drugs is a matter of concern.

There is a general consensus that treatment in ET has to be risk-adapted. It is evident that treatment is indicated in symptomatic patients and in asymptomatic patients with a history of a major thrombotic or bleeding episode (Cortelazzo et al. 1995, Tefferi et al. 2000, Tefferi 2001a, Schafer 2004). Platelet lowering treatment decreases the incidence of thrombohaemorrhagic

complications, even though it does not abolish them totally, and the patients may experience thrombotic complications with only slightly elevated platelet counts (Hehlmann et al. 1988, Fenaux et al. 1990, Pearson 1991, Cortelazzo et al. 1995, Regev et al. 1997, Lengfelder et al. 1998). There are several risk-adjusted treatment strategies dividing the patients into low-, intermediate- or high-risk patients based on their age, history of bleeding or thrombosis, and platelet count (Barbui and Finazzi 1999, Michiels 1999, Tefferi 1999, Tefferi 2001a, Harrison et al. 2003, Barbui et al. 2004b, Schafer 2004, Harrison et al. 2005b).The efficacy of platelet lowering treatment in high-risk patients, i.e. patients aged more than 60 years or with a history of previous thrombosis, has been prospectively demonstrated by Cortelazzo et al. (1995). Limited data is available on the thrombohaemorrhagic risk in younger asymptomatic patients, and the optimal approach is unknown. Until now, the only prospective, controlled study in low-risk ET patients showed similar incidences of thrombohaemorrhagic complications in the ET patients and the controls, and a conservative approach was suggested for these patients (Ruggeri et al. 2003). Also in some other studies a low incidence of thrombohaemorrhagic complications in low-risk patients has been reported (Randi et al. 1999a, Tefferi et al. 2001b). The role of cardiovascular risk factors in treatment decisions is controversial (Cortelazzo et al. 1990, Watson and Key 1993, Bazzan et al. 1999, Tefferi 2001a).

ET patients have been treated with a variety of cytotoxic drugs including melphalan, busulphan, chlorambucil, and radioactive phosphorus (^{32}P), but the leukaemogenic potential of these drugs is well documented (Berk et al. 1986, van de Pette et al. 1986, Brandt and Anderson 1995, Balan and Critchley 1997). Hydroxyurea is the only platelet lowering drug, the efficacy of which in the prevention of thrombohaemorrhagic complications has been prospectively shown in a randomized study (Cortelazzo et al. 1995). However, the leukaemogenic potential of hydroxyurea is a matter of debate. Some non-randomized studies have indicated an increased risk of secondary leukaemia associated with the long-term use of hydroxyurea in ET (Weinfeld et al. 1994, Furgerson et al. 1996, Liozon et al. 1997). In larger studies the incidence of leukaemic transformation in ET patients treated with hydroxyurea as the only treatment has ranged from 0 to 5 % (Nand et al. 1996, Murphy et al. 1997, Sterkers et al. 1998, Finazzi et al. 2000). Mutagenic studies have not supported the leukaemogenity of hydroxyurea (Hanft et al. 2000). Many authors agree that hydroxyurea as a single agent carries only a slightly elevated, if any, risk of leukaemic transformation (Tefferi 2001a, Barbui 2004a, Harrison 2005b). However, the sequential use of hydroxyurea with other cytotoxic drugs increases markedly the risk of leukaemic transformation

(14-33 % of the patients) (Murphy et al. 1997, Sterkers et al. 1998, Finazzi et al. 2000). The need for multiple treatments may, on the other hand, reflect an aggressive form of ET and a tendency more likely to transform.

During recent years the concern of leukaemogenesis has encouraged the use of non-leukaemogenic platelet lowering drugs including interferon alfa (IFN) and anagrelide, especially in younger patients. IFN is a biological response modifier which has myelosuppressive activity and an ability to antagonize the action of the platelet-derived growth factor without any mutagenic risk (Lengfelder et al. 1996, Elliot and Tefferi 1997, Barbui et al. 2004a). It normalizes the platelet count in approximately 80-90 % of the patients, but side effects impairing the quality of life necessitate drug cessation in a quarter of ET patients (Lengfelder et al. 1996). Anagrelide acts by inhibiting megakaryocyte maturation and platelet aggregation (Mazur et al. 1992, Petit et al. 1997, Tomer 2002). It reduces effectively the platelet count in 70-90 % of the patients (Silverstein et al. 1992, Petrides et al. 1998, Birgegård et al. 2004, Fruchtman et al. 2005). In some reports anagrelide has turned out to be relatively well tolerated (Silverstein et al. 1992), but in two prospective studies even 37-50 % of the patients discontinued the treatment because of neurological, gastrointestinal or cardiac side-effects (Mills et al. 1999, Birgegård et al. 2004). Leukaemic transformation has not been observed in long-term use (Storen and Tefferi 2001, Fruchtman et al. 2005). Recently, however, accelerated fibrotic transformation in ET patients using anagrelide has been reported (Harrison et al. 2005c). Up till now there is no controlled evidence of the efficacy of anagrelide or IFN to prevent thrombohaemorrhagic complications, though it is presumable that adequate lowering of the platelet count also prevents complications. However, a recent randomized study (PT1-trial) showed that the combination of anagrelide and low-dose acetylsalicylic acid (ASA) was not as effective as that of hydroxyurea and ASA in preventing arterial thrombotic complications and it also increased the risk of major bleedings (Harrison et al. 2005c).

Due to the risk of aggravating the bleeding tendency, the use of ASA in MPDs was long controversial (Schafer 1984). However, thromboxane A_2 (TXA_2) biosynthesis is increased in ET and it can be suppressed with low-dose ASA (50-100 mg per day) (Rocca et al. 1995). There is evidence that enhanced formation of TXA_2 by platelets reflects platelet activation *in vivo*, which precedes the development arterial thrombosis in ET (van Genderen et al. 1999b). ASA relieves effectively microvascular disturbances of the peripheral and cerebral circulation and reduces

recurrences of thrombotic events in symptomatic patients (van Genderen et al. 1995, Griesshammer et al. 1997, van Genderen et al. 1997a, Michiels 1999, Randi et al. 1999b). The role of low-dose ASA in the primary prevention of thrombosis in ET is a matter of debate. Recently the efficacy and safety of low-dose ASA to prevent thrombotic complications was shown in PV (Landolfi et al. 2004). The use of low-dose ASA in ET has been recommended in high-risk patients together with the use of platelet lowering drugs, but the benefit of the ASA treatment in low- and intermediate-risk patients is unclear (Tefferi 2001a, Barbui et al. 2004b, Elliott and Tefferi 2005). However, according to some management guidelines, ASA is recommended to all patients with ET (Campbell and Green 2005). There are only case-reports concerning the use of newer antiplatelet agents, such as clopidogrel, in patients with ET (Klinzing et al. 2001, Mosso et al. 2004).

Prognosis

ET may transform to myelofibrosis or acute leukaemia as part of the natural course of the disease. Knowledge of myelofibrotic transformation is scarce, but in the study of Cervantes et al. (2002) myelofibrosis was observed in 8 % at ten years and in 15 % at 15 years. In untreated patients the incidence of progression to acute leukaemia has been reported to be a sporadic phenomenon (<5 %) (Fenaux et al. 1990, Sterkers et al. 1998, Cervantes et al. 2002, Barbui 2004a, Fruchtman 2004). Leukaemic transformation is, however, significantly associated with cytotoxic treatment (see above) and cytogenetic abnormalities (Löfvenberg et al. 1990).

Studies of the prognosis of ET are relatively few and controversial. Rozman et al. (1991) and Passamonti et al. (2005b) reported that the actuarial survival probability of ET patients was similar to that of the control population, whereas the Olmsted County and the Copenhagen County studies showed significantly lower survival for ET patients (Mesa et al. 1999, Jensen et al. 2000a). In younger patients a fourfold relative risk of mortality was found by Bazzan et al. (1999), whereas Tefferi et al. (2001b) reported that young females with ET show a similar survival compared to control population.

Pregnancy in ET

Knowledge of pregnancies in ET patients is limited, and many reports contain small numbers of pregnant patients. Only a few reports with a relatively large patient number are available (Beressi et al. 1995, Cincotta et al. 2000, Wright and Tefferi 2001). In some previous studies the outcome of pregnancy has mostly been successful (Jones et al. 1988, Linares et al. 1988, Beard et al. 1991, Randi et al. 1994, Randi et al. 2000, Chow et al. 2002), but high rates of miscarriages have also been reported (Falconer et al. 1987, Radaelli et al. 1994, Beressi et al. 1995, Pagliaro et al. 1996, Bangerter et al. 2000, Cincotta et al. 2000). The risk of first-trimester abortion is increased, and placental insufficiency may lead to foetal growth retardation and intrauterine foetal death. According to two recent reviews, live birth can be expected in 50-59 % of the pregnancies (Vantroyen and Vanstraelen 2002, Griesshammer et al. 2003). Pregnancies may also be complicated by pre-eclampsia and preterm delivery. No prognostic factors for pregnancy complications have been found (Beressi et al. 1995, Cincotta et al. 2000, Wright and Tefferi 2001).

The reports of the results of treatment before or during the pregnancy have been conflicting (Falconer et al. 1987, Chow et al. 1992, Randi et al. 1994, Beressi et al. 1995, Pagliaro et al. 1996, Bangerter et al. 2000, Cincotta et al. 2000, Randi et al. 2000, Wright and Tefferi 2001, Ruggeri et al. 2003). The use of low-dose ASA for the prevention of pregnancy complications was advantageous in the studies of Cincotta et al. (2000), Randi et al. (2000), and Bangerter et al. (2001). In the literature review of Griesshammer et al. (2003) the live-birth ratio among the patients treated with ASA alone or in combination was 68 % but in the untreated patients only 48 %. Pagliaro et al. (1996) combined ASA successfully with low-molecular-weight heparin in a study of seven patients. However, no benefits of the ASA therapy in the prevention of miscarriages were observed in the largest published reports with 34 and 43 pregnancies from the Mayo Clinic (Beressi et al. 1995, Wright and Tefferi 2001). In most studies the number of ET patients treated with IFN during pregnancy has been too small for any definite conclusions (Feelders et al. 1994, Perez-Encinas et al. 1994, Thornley and Manorahan 1994, Vianelli et al. 1994, Williams et al. 1994, Delage et al. 1996, Diez-Martin et al. 1996, Frezzato et al. 1996, Milano et al. 1996, Pulik et al. 1996, Shpilberg et al. 1996, Schmidt et al. 1998, Cincotta et al. 2000, Vantroyen and Vanstraelen 2002). Hydroxyurea should be avoided during pregnancy due to the risk of teratogenic effects, though there have been reports of successful pregnancies after

exposure to hydroxyurea (Thauvin-Robinet et al. 2001). Anagrelide crosses the placental barrier and may lead to foetal thrombocytopenia. There are no adequate studies of the use of anagrelide during pregnancy, but, according to general view, the use of anagrelide in pregnant women should be avoided.

THE AIMS OF THE PRESENT STUDY

The purpose of the present thesis was to study the findings at diagnosis and the diagnostic procedures in ET, to evaluate the clinical course of ET in Finnish patients, and to identify predictive risk factors which could be used in treatment decisions.

The specific aims were as follows:

- to assess the causes leading to diagnostic evaluation for ET and the presence of positive diagnostic findings (I).
- to study the occurrence of thrombotic and bleeding complications during the course of the disease and their association with age, sex, platelet count and cardiovascular risk factors (III).
- to analyse the diagnostic and predictive value of the *in vitro* colony formation of haematopoietic progenitors and platelet function tests (I and IV).
- to clarify the transformation of ET to other myeloproliferative disorders (II).
- to analyse the clinical course and outcome of pregnancies in ET as well as the effect of treatment on the outcome of pregnancy (V).

PATIENTS AND METHODS

Patients

The consecutive patients diagnosed as having ET during the years 1980-1995 were identified from the databases of the Helsinki University Central Hospital, Helsinki Municipal Hospitals, Jorvi Hospital, Peijas Hospital, and Central Hospital of Kanta-Häme. In addition, data of ET patients with pregnancies was asked from all Finnish university and central hospitals where patients with haematological diseases were treated (V). All hospital records of the patients were reviewed. Only the patients fulfilling the modified diagnostic criteria (below) of the PVSG (Murphy et al. 1997) on re-evaluation were included in the study. The bone marrow aspirates of 128 patients were re-examined (E.J / S-E.J).

The diagnosis of ET was established if the following criteria were fulfilled:
- platelet count over 600 x 10^9/l for at least six months
- no erythrocytosis and exclusion of iron deficiency
- no Philadelphia chromosome (the karyotype analysis was not available in many patients with no or minimal leukocytosis)
- no leukoerythroblastic blood picture or morphological abnormalities of erythrocytes compatible with myelofibrosis
- no known cause of reactive thrombocytosis

For thrombocytosis a shorter follow-up than six months was accepted if cytoreductive therapy had been started due to symptoms considered to be related to thrombocythaemia. Adequate iron stores were verified by normal serum iron, transferrin, and ferritin concentrations or by the presence of adequate iron stores in bone marrow. In 15 patients iron deficiency was corrected with iron therapy.

Study I. The diagnostic evaluation and the clinical and laboratory data at diagnosis were retrospectively analysed in all 170 patients with ET fulfilling the diagnostic criteria on re-evaluation. At the diagnosis of ET the median age was 52 years (range 19-88 years), 54 years (range 21-88

years) in males and 50 years (range 19-85 years) in females. At the time of the diagnosis of ET the median platelet count was 885 x 10^9/l (range 496-2175 x 10^9/l).

Study II. With the median follow-up time of 63 months (range 11-313 months) the development of erythrocytosis was evaluated in the patient population described above.

Study III. The occurrence of thrombotic and bleeding complications and the history of vascular risk factors including smoking, hypertension, diabetes mellitus, and hypercholesterolaemia, were registered from the patient data. The data of vascular risk factors was available of 132 patients (70 females and 62 males). All these patients were interviewed with a questionnaire. At the diagnosis the median age was 51 years (range 19-88 years) and the median platelet count 866 x 10^9/l (range 496-2175 x 10^9/l). During the follow-up the median peak platelet count was 1000 x 10^9/l (range 600-2800 x 10^9/l). The median follow-up time of the patients with one or more vascular risk factors was 65 months (range 12-291 months) and that of those without risk factors 74 months (range 11-313 months).

Study IV. *In vitro* cultures of erythroid and megakaryocytic progenitors were analysed in 154 patients and platelet aggregation studies in 55 patients to assess the predictive value of spontaneous colony formation and platelet function on the occurrence of thrombotic and bleeding complications. The median follow-up of this patient cohort was 60 months (range 11-313 months). At the time of the diagnosis the median age was 49 years (range 19-88 years), and 55 patients were younger than 45 years of age. Progenitors from bone marrow only were cultured in 106 patients, from peripheral blood only in 17 patients, and from both bone marrow and peripheral blood in 31 patients.

The *in vitro* cultures of haematopoietic progenitors were performed as part of the diagnostic evaluation in 134 patients (87 %) and during the follow-up in 20 patients with the median of 53 months (range 11-168 months) after the diagnosis. One hundred and forty-seven patients had not received any platelet lowering treatment before the *in vitro* cultures of haematopoietic progenitors. In two patients platelet lowering treatment had been discontinued shortly prior to the cultures and in the other five patients several years before that (range 2-13 years). The proportion of the patients treated with platelet lowering treatment or ASA before the appearance of the first major complication was similar in the patients with a normal haematopoietic progenitor growth pattern and

in those with spontaneous colony formation (8 % vs 10 % and 30 % vs 21 %, respectively). Platelet function studies were carried out in 55 patients, in 30 patients at the time of the diagnosis and in 25 patients during the follow-up with the median of 59 months from the diagnosis (range 4-216 months). None of the patients was on ASA therapy at the time of the platelet function studies or during the preceding ten days.

Study V. The hospital records of 16 females with 40 pregnancies at five Finnish hospitals during the years 1980-1998 were reviewed. In six patients the diagnosis of ET was established before the first pregnancy (median of 69 months, range 3-120 months before the pregnancy). In ten patients thrombocytosis was detected for the first time during a pregnancy. In two of these a pregnancy ending in miscarriage within the preceding six months was also included in the present analysis. The median age of the patients at the diagnosis of ET was 27 years (range 19-38 years). The median initial platelet count was 1082 x 10^9 /l (range 605-2498 x 10^9/l), and the median follow-up from the diagnosis was 78 months (range 17-168 months).

Methods

Evaluation of the clinical features and risk factors (studies I - V)

Thrombohaemorrhagic complications were classified as follows: Arterial complications included disturbances in the cerebral circulation (cerebral infarction, transient ischaemic attack), myocardial ischaemia (angina pectoris, myocardial infarction), claudication, peripheral gangrene, and arterial thromboembolism. Transient disturbances of the peripheral circulation (erythromelalgia, Raynaud's phenomenon) and the cerebral circulation (visual disturbances, dizziness, migraine, epilepsy) were classified as microvascular symptoms. Venous thrombosis consisted of deep venous thrombosis of the extremities diagnosed by ultrasound or venography, pulmonary embolism, intra-abdominal venous thrombosis, and recurrent thrombophlebitis. Major bleeding complications included bleedings requiring transfusion, hospitalization or surgical treatment, and gastrointestinal, intracranial and excessive procedure-related bleedings.

The vascular risk factors were defined as follows: The patients who smoked during the study or had smoked within one year preceding the study period were classified as smokers. The patients who had stopped smoking more than one year before the diagnosis of ET and those who had

never smoked were regarded as non-smokers. Hypertension was recorded as a risk factor in the patients on antihypertensive treatment and in those who had repeatedly systolic blood pressure higher than 160 mmHg or diastolic blood pressure higher than 95 mmHg. Hypercholesterolaemia was defined as serum total cholesterol higher than 6.5 mmol/l and low density lipoprotein cholesterol higher than 3.5 mmol/l. Diabetes mellitus was defined as fasting blood glucose higher than 6.7 mmol/l repeatedly.

A questionnaire was sent to all patients in Study III. The questions concerned symptoms and complications of the disease, presence of cardiovascular risk factors, history of pregnancies and operations, and medication.

Treatment

The choice of therapy was based on the clinical judgement of the physician. Cytotoxic treatment was avoided at least in younger patients unless they had thrombohaemorrhagic complications before or at the diagnosis of ET. Due to the retrospective nature of the present study, the treatment varied during the study period depending on the therapy generally recommended at the time of diagnosis as well as on the local treatment practice. During the study period the treatment strategies changed, especially with the reduced use of busulphan and increased use of IFN in young patients. Overall, 99 patients received platelet lowering treatment which included busulphan (n = 49), hydroxyurea (n = 39), ^{32}P (n = 13), and IFN (n = 25). The treatment was started in 85 % of the patients because of a thrombotic or bleeding complication and in 15 % because of a high platelet count or preoperatively. The patients younger than 60 years were treated as frequently as older patients (58 % vs. 61 %, respectively). ASA was given to 98 patients.

Cultures of haematopoietic progenitors

Megakaryocyte progenitors were cultured according to the method of Messner et al. (1982). For the cultures of progenitors from the bone marrow 1-2 ml of marrow was aspirated and diluted in Iscove's Modified Dulbecco's Medium (IMDM). For the cultures of peripheral blood precursors 10-20 ml heparinized venous blood was collected. Mononuclear cells were isolated by centrifugation, washed, and resuspended in IMDM. The culture medium consisted of 30 %

plasma from a patient with aplastic anaemia with 5 % phytohaemagglutinin-stimulated leukocyte conditioned medium (PHA-LCM) (stimulated colony formation) or 30 % normal human plasma without PHA-LCM (spontaneous colony formation), 5 x 10^{-5}M 2-mercaptoethanol, and 0.9 % methyl cellulose in IMDM. The final mononuclear cell concentration was 2 x 10^5/ml. Megakaryocytic colonies containing at least five cells with a non-granular, translucent cytoplasm and a distinct cell border of high refractivity were scored on the 14th day of culture.

Erythroid and granulocyte-macrophage progenitors were cultured as described by Iscove et al. (1974) with the modification of Guilbert and Iscove (1976). For the cultures of bone marrow progenitors 1-2 ml of marrow was aspirated and diluted in IMDM, red cells were removed by sedimentation with 10 % dextran, the leukocyte-rich supernatant was collected, and the cells were washed. For the cultures of peripheral progenitors 10-20 ml of peripheral blood was collected, mononuclear cells were isolated by Ficoll-Isopaque gradient, washed, and resuspended in IMDM. The culture medium consisted of 0.8 % methyl cellulose, 20 % foetal calf serum, 1 % delipidated and deionized bovine serum albumin, 10^{-4} mercaptoethanol, 310 ug/ml fully iron saturated human transferrin, 20 % human leukocyte conditioned medium prepared in IMDM, and IMDM. The number of mononuclear cells in the cultures was 2 x 10^5/ml. Colony formation of erythroid progenitors was stimulated with erythropoietin (2 U/ml) and colony formation of granulocyte-macrophage progenitors with leukocyte feeder layers as described by Pike and Robinson (1970). Erythroid colonies of at least eight cells were scored on the seventh day of culture for CFU-E and colonies with at least three subclusters on the 14th day of culture for BFU-E. The cultures of granulocyte-macrophage progenitors (CFU-GM) were scored on the seventh and 14th day for colonies containing 40 or more cells.

Platelet function studies

Platelet aggregation tests were carried out with the method described by Ludlam (1994) at the laboratory of the Finnish Red Cross Blood Transfusion Service. Platelet-rich plasma was diluted with autologous platelet-poor plasma to the platelet concentration of 250 x 10^9/l. The following aggregation-inducing agents were used: ADP (1 μg/ml, 3 μg/ml, 6 μg/ml), adrenaline (0.9 μg/ml, 9 μg/ml), arachidonic acid (0.6 mmol/l, 1.2 mmol/l), ristocetin (1.25 mg/ml, 1.5 mg/ml), and collagen (0.42 μg/ml, 0.84 μg/ml).

Cytogenetic studies

Conventional cytogenetic studies with G-banding were performed in 76 patients at the time of the diagnosis.

Statistical analysis

The statistical differences in the distribution of continuous variables were analysed with the Mann-Whitney U-test. The Chi-Square test with continuity correction or Fischer's exact test was used to compare the groups of the categorical data. The logistic regression model with forward selection was used in a multivariable analysis to test the risk factors. The survival time was estimated using the method of Kaplan and Meier. All statistics were performed with SPSS software for Windows 95. All p-values are two-tailed.

RESULTS

Diagnostic findings (I, IV)

Clinical features at diagnosis (I)

Of the patients 97 were female and 73 male, with an F: M ratio of 1.3:1. The median age of the patients was 52 years (range 19-88 years). The peak incidence among females was in the age group of 40-49 years and among males in the age group of 60-69 years (Fig. 1). The females showed another less clear increase of incidence in the age group of over 70 years.

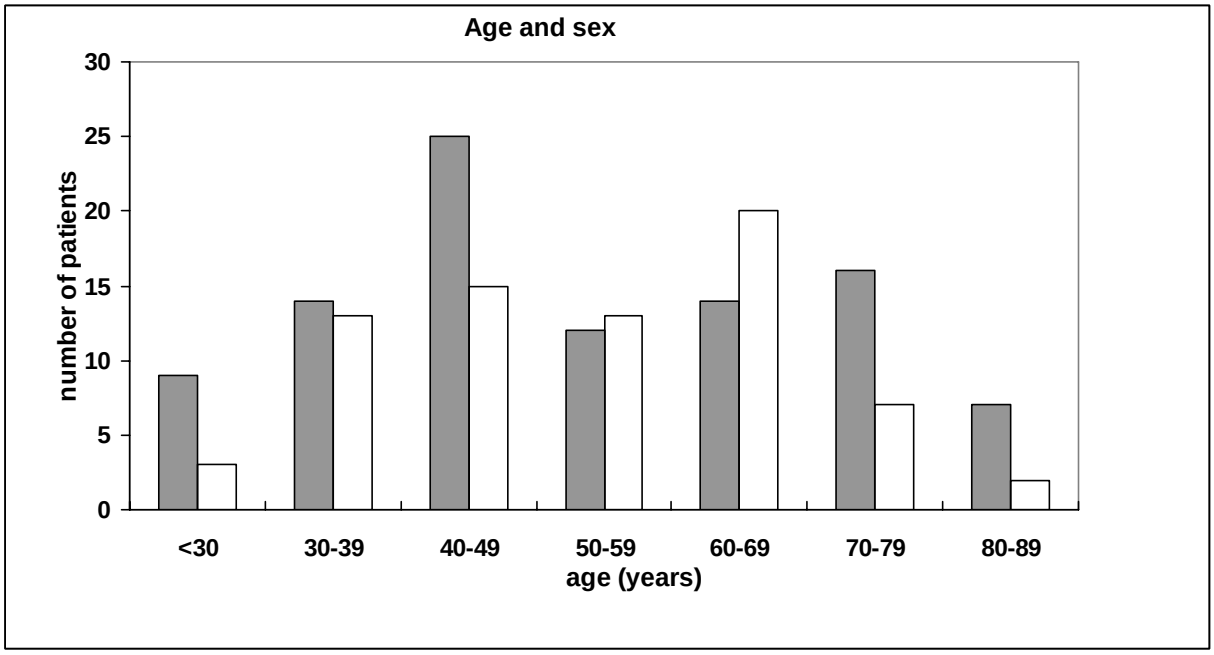


Figure 1. The age and sex distribution of the patients at the time of diagnosis. The grey and white bars represent females and males, respectively.

Thrombocytosis was a chance finding in 65 % of the patients (n = 111), in 74 % of the females (n = 72) and in 53 % of the males (n = 39), while in 35 % of the patients (n = 59) symptoms were the reason for diagnostic evaluation. However, the past history of 37 "asymptomatic" patients revealed complications or symptoms which are known to be seen in ET. Thus, finally only 74 patients (44 %) had no history of symptoms caused by ET. The females were significantly (p = 0.002) more often asymptomatic at diagnosis than the males, and the difference was seen in all

age groups (< 40 years: 78 % vs. 56 %; 40-59 years: 78 % vs. 43 %; ≥ 60 years: 74 % vs. 59 %, respectively).

The complications at diagnosis are summarised in Table 7. Forty-eight patients (28 % of all patients and 50 % of those with complications or symptoms) had experienced altogether 70 thrombotic complications, nearly half of which were major disturbances of the cerebral circulation. Three patients younger than 35 years of age had had an acute myocardial infarction. Thirty-nine patients (23 %) showed bleeding complications which caused only minor problems. Both thrombotic and haemorrhagic complications were detected in nine patients (5 %).

Table 7. The symptoms and complications of patients with ET at or preceding the diagnosis. The number of patients in whom the symptom or complication led to the diagnosis is given in parenthesis. The median and range of the platelet count (PLTx10^9/l) and the median age at the time of complication are shown.

	n[1]	PLT Med	Range	Age Med	Range
No symptoms	**74**	929	500-2124	54	21-85
Thrombosis	**48**				
Arterial:	**43**				
cerebral infarction	18 (16)	617	262-1800	51	24-79
transient ischaemic attack	11 (9)	717	374-1100	53	32-69
myocardial infarction / unstable angina pectoris	10 (6)	714	534-1288	47	27-85
claudication	6 (4)	761	496-1000	59	34-88
spontaneous abortion / stillborn baby	3 (3)	749	550-1320	32	24-38
gangrene of toe	2 (0)				34-48
bone infarction	1 (1)		788		41
brachial artery embolism	1 (0)		582		46
optic nerve ischaemia	1 (0)		694		80
Venous:	**7**				
deep venous thrombosis	5 (4)	775	262-2000	52	29-75
pulmonary embolism	2 (2)		262-425		29-50
recurrent thrombophlebitis	1 (1)		733		28
portal vein thrombosis	1 (1)		986		36
Microvascular	**36**				
peripheral symptoms[2]	20 (10)	848	606-1729	51	39-88
dizziness	10 (7)	806	620-1245	51	27-76
visual disturbance	5 (1)	960	623-1547	47	45-53
headache	4 (1)	607	507-1060	46	41-51
migraine	2 (0)		620-1392		40-51
grand mal epilepsy	1 (1)		675		58
confusion	1 (1)		679		70
Haemorrhages	**39**				
bruising	12 (3)	826	534-1340	47	32-72
postoperative	11 (6)	810	520-2700	54	18-71
gynecological	5 (0)	1024	743-1392	40	32-49
gastrointestinal	4 (3)	927	776-1225	55	26-72
wound bleeding	4 (0)	992	679-1320	46	38-63
nasal	4 (1)	1004	679-1729	65	46-81
gingival	3 (2)	794	728-1410	47	42-54
haemoptysis	2 (1)		552-741		34-40
urethral	2 (1)		622-844		67-73
testicular	1 (1)		944		60

[1] = number of patients with the given symptom or complication
[2] = erythromelalgia, paraesthesia, Raynaud's phenomenon, acral cyanosis

Laboratory and radiological findings (I)

At the time of the diagnosis the median platelet count of the 170 patients was 885 x 10^9/l and the range 496-2175 x 10^9/l. The distribution of the platelet counts at diagnosis and the proportion of the patients with leukocytosis are shown in Table 8. In the bone marrow examination the most frequent abnormality was slight or moderate megakaryocytic hyperplasia, seen in 79 % of the aspirates and in 93 % of the biopsies (Table 8). In 80 % of the aspirates a variable number of large megakaryocytes with hyperlobulated nuclei were present. The bone marrow cellularity was normal in two thirds of the biopsies, and in 83 % of the evaluable aspirates it was estimated to be normal. In the rest of the biopsies and aspirates the cellularity was increased. Twenty-six per cent (32/121) of the patients examined either with ultrasound or tomography showed an enlarged spleen. The spleen was palpable in only five patients. Two patients had been splenectomized for diagnostic purposes six and 14 months before the diagnosis of ET, respectively.

Table 8. The proportion of patients with abnormal findings at diagnosis. Total number of patients studied with the given test or examination is in parenthesis.

	% of patients studied
Platelets x 10^9/l (170)	
lower than 600	4
600-999	59
1000-1499	29
more than 1500	8
Leukocytosis > 10 x 10^9/l (170)	32
Bone marrow aspirate / biopsy (154 / 31)	
increased cellularity	17 / 33
increased number of megakaryocytes	79 / 93
abnormal megakaryocyte morphology	80 / 55
Splenomegaly (ultrasound or tomography) (121)	26
Abnormal platelet function (36)	83
Karyotype abnormality (76)	5
Spontaneous colony formation (134)	74

Cultures of haematopoietic progenitors (I, IV)

The erythroid (CFU-E and BFU-E) and megakaryocyte (CFU-Meg) progenitors of 154 patients were cultured *in vitro* by the methyl cellulose assay. At the time of the cultures 134 patients had a newly diagnosed disease, whereas 20 patients had had ET diagnosed 1-13 years before the

cultures. Either erythroid or megakaryocytic spontaneous colony formation was detected in 74 % of the patients (n = 114), whereas 26 % (n = 40) showed a normal growth pattern (Table 9). Of the patients 57 % (n = 88) had spontaneous erythroid colony formation and 59 % (n = 91) spontaneous megakaryocyte colony formation. Both megakaryocyte and erythroid spontaneous colony growth was shown by 42 % of the patients (n = 65). Spontaneous growth only in the megakaryocyte or erythroid cultures was seen in 17 % (n = 26) and 15 % (n = 23) of the patients, respectively. The presence of spontaneous colony formation was not statistically significantly different in the patients with blood progenitor cultures only when compared to the remaining patients.

The platelet count at the time of the cultures had no effect on the growth pattern. The median platelet count was 843 x 10^9/l (range 464-2498 x 10^9/l) among the patients with spontaneous colony formation and 1014 x 10^9/l (range 531-1757 x 10^9/l) among those with normal colony growth. The presence of spontaneous colony formation was similar in the males (79 %) and the females (70 %).

Table 9. Megakaryocytic and erythroid colony formation in 154 patients with ET.

		Spontaneous megakaryocytic colony formation		
		Yes	No	Total
Spontaneous erythroid colony formation	Yes	65 (42%)	23 (15%)	88 (57%)
	No	26 (17%)	40 (26%)	66 (43%)
	Total	91 (59%)	63 (41%)	

Platelet function (I, IV)

The platelet function tests were performed in 36 patients at the time of the diagnosis or within a year after the diagnosis. Eighty-three per cent of them had defective aggregation. Of all 55 patients investigated, 75 % (n = 41) showed abnormalities in the platelet function tests (Table 10). The most common abnormality was defective aggregation induced by adrenaline (n = 32). No correlation was found between the platelet aggregation responses and the colony formation of

the haematopoietic progenitors (Table 10). Of the patients studied 51% (n = 28) showed defective platelet aggregation response with more than one inducing agent. The sex and age distribution of the patients with normal and abnormal platelet function was similar.

Table 10. Platelet function tests in 55 patients with ET and the proportions of the patients with spontaneous colony formation.

	No. of patients n = 55	Proportion with spontaneous growth
Normal aggregation	14 (25 %)	10 / 14
Hypoaggregation	41 (75 %)	26 / 41
adrenaline	32	
collagen	21	
ristocetin	16	
ADP	14	
arachidonate	2	

Cytogenetic features (I)

At diagnosis conventional cytogenetic studies revealed an abnormal karyotype in four out of 76 patients (5 %): 7q+; 3p-; 20q-,9+; and a structural abnormality in chromosome 16.

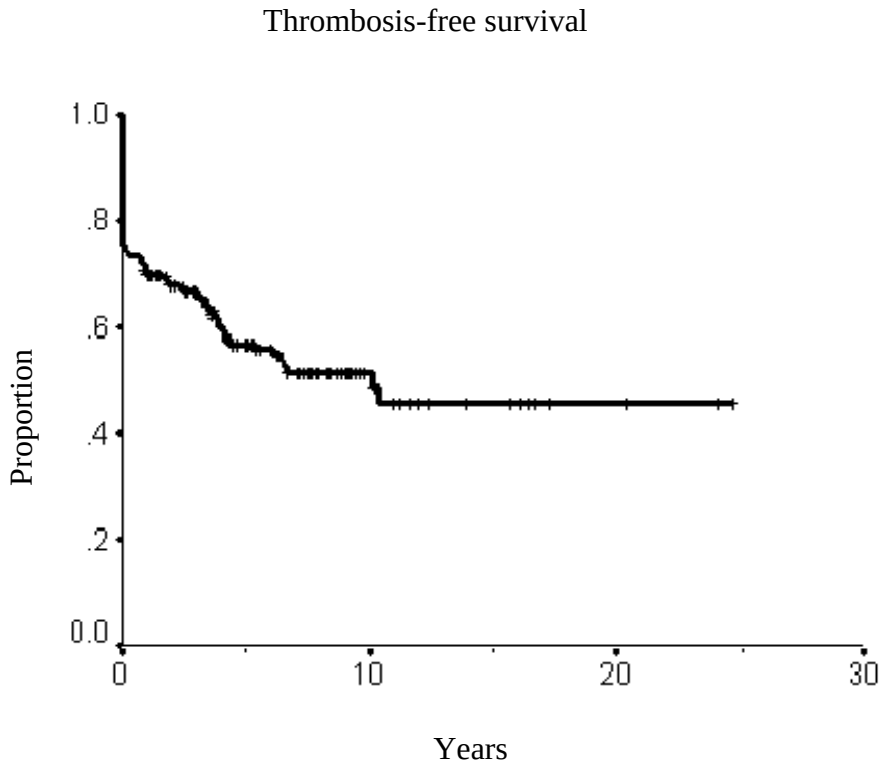
Complications and transformation (I-IV, unpublished data)

During the course of the disease, with the follow-up of 63 months, only 22 % of the 170 patients remained asymptomatic. Either at diagnosis or during the follow-up 76 patients (45 %), including five patients with a pregnancy loss, experienced altogether 144 thrombotic events. Only 20 % of all thrombotic complications were venous. Of the patients 21 % (n = 35) had a major bleeding complication. Either thrombosis or major bleeding occurred in 56 % of the patients (n = 95). Half of the patients had microvascular symptoms, the most common of which were disturbances of the peripheral circulation. Survival free from thrombosis and major bleedings is shown in Figure 2.

During the follow-up 24 patients (14 %) died. In 15 patients death was caused by thrombotic or haemorrhagic complications: cerebral infarction (n = 6), coronary artery disease or myocardial infarction (n = 4), pulmonary embolism (n = 4), and subdural bleeding (n = 1). The causes of death likely to be unrelated to ET included non-haematological malignancy (n = 3), pneumonia (n = 2), non-Hodgkin lymphoma (n = 1), aortic valve stenosis (n = 1), renal insufficiency (n = 1), and tetraplegy (n = 1). The probability of a ten-year survival was 90 % (Fig. 3).

Transformation to MDS (refractory anaemia) occurred in one patient. The patient died of ventricular fibrillation within a year. No transformation to acute leukaemia occurred. Three patients transformed to myelofibrosis with collagen fibrosis of the marrow and leukoerythroblastic blood picture after four to six years of follow-up.

Of the patients 6.5% (11/170) developed a constant erythrocytosis at the median of 29 months (range 12-138 months) after the diagnosis. The time from the discovery of thrombocytosis to the appearance of erythrocytosis ranged from 15 to 146 months (median 48 months). Despite erythrocytosis, in two patients the haemoglobin and haematocrit values remained within the normal range. In the other patients also the haemoglobin and haematocrit values exceeded the upper limit of the normal range. No significant differences could be found in the findings at diagnosis between the patients who later developed erythrocytosis and those who did not. The erythrocyte count, haemoglobin concentration, and haematocrit of the patients with later erythrocytosis were slightly, but not significantly, higher than those of the other patients. The cellularity and the proportion of erythropoiesis of the initial bone marrow aspirates as well as the presence of splenomegaly (14 % vs. 25 %, respectively) were similar in the patients with later erythrocytosis and in those with a stable disease. All ten patients with later erythrocytosis whose progenitors were cultured in the non-erythrocytic phase showed spontaneous erythroid colony formation compared to only 54 % of the patients with stable ET. Spontaneous megakaryocytic colony formation was seen in 80 % (8/10) of the patients with later erythrocytosis and in 57 % of those with a stable disease. The frequency of symptoms at diagnosis or after the development of erythrocytosis did not differ between the two groups.



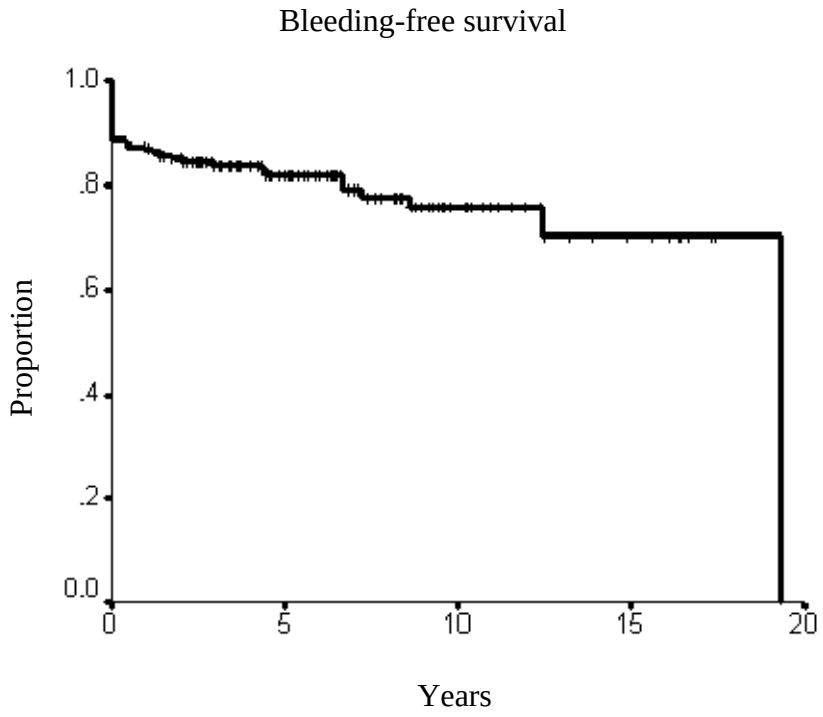


Figure 2. Thrombosis-free and major bleeding-free survival of 170 patients with ET.

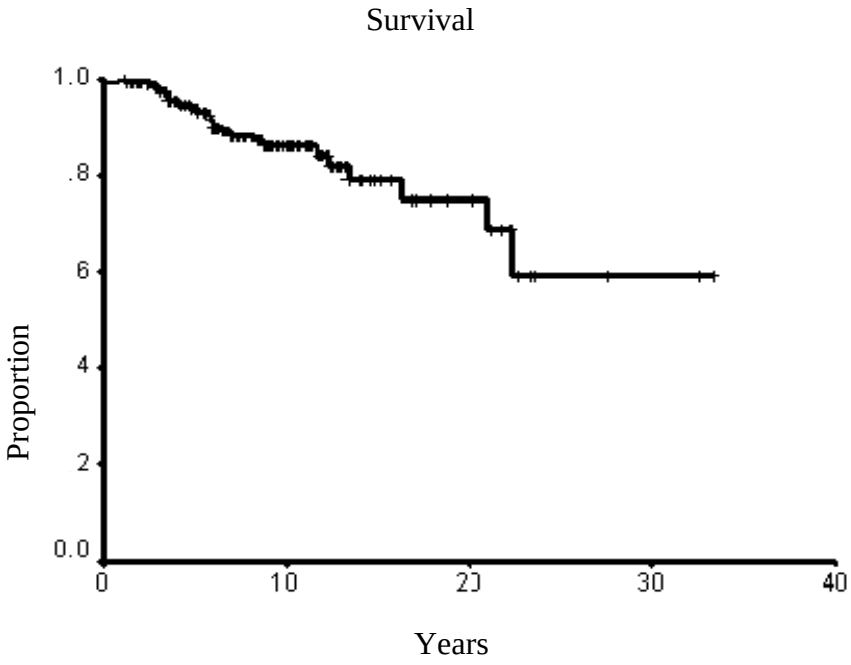


Figure 3. Overall survival of 170 patients with ET.

Prognostic factors for vascular complications

Platelet count and age of the patients (I, III, IV, unpublished data)

The risk of thrombotic or bleeding complications did not correlate with the platelet count. Of the thrombotic episodes 79 % occurred with a platelet count lower than 1000 x 10^9/l, 30 % with a platelet count lower than 600 x 10^9/l, and 14 % with a platelet count lower than 450 x 10^9/l. Similarly, the majority of the major bleedings (61 %) occurred with a platelet count lower than 1000 x 10^9/l.

The thrombotic complications did not correlate with age. One thrombotic complication or more were shown by approximately 30-40 % of the patients in each age group (Fig. 4). The risk of major bleedings was increased in the elderly patients. Twenty-eight per cent of the patients older than 60 years but only 14 % of the younger ones experienced a major bleeding episode (p = 0.03).

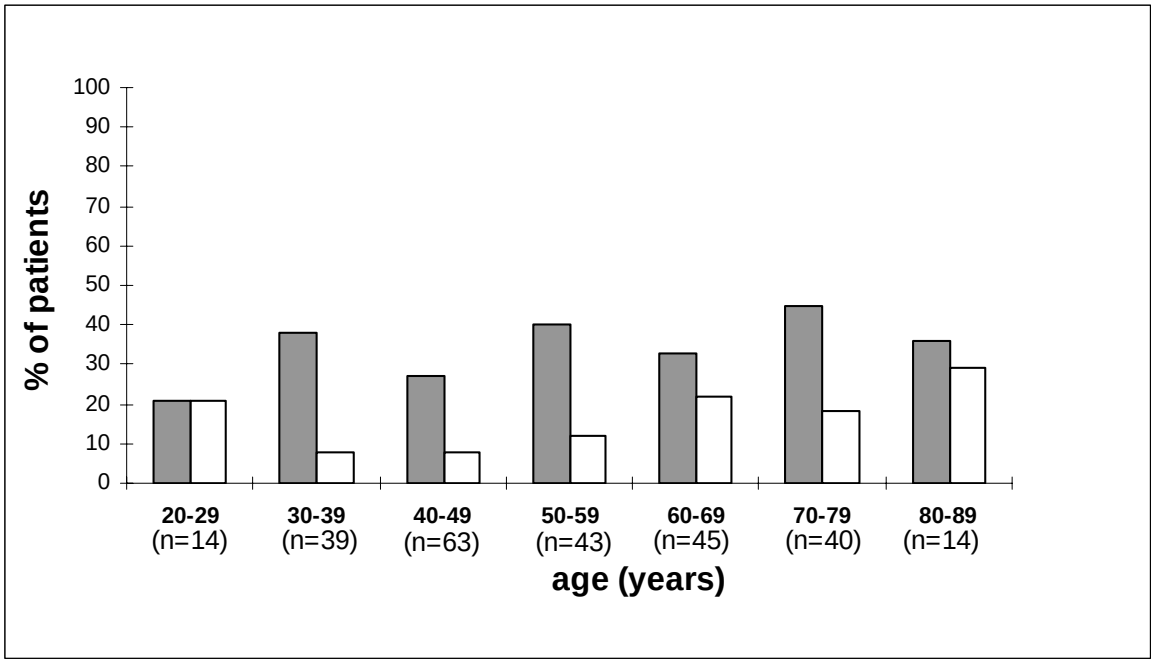


Figure 4. The proportion of the patients with thrombosis or major bleeding in the different age groups. The grey and white bars represent thrombotic and major bleeding complications, respectively.

Sex (I, III, IV)

In multivariate analysis the male gender had a significant, independent prognostic impact on the risk of thrombotic complications, especially on the risk of arterial thrombosis. Of the males 58 % (36/62), whereas only 24 % (17/70) of the females, experienced arterial thrombosis (p = 0.0001). Males showed all types of arterial complications more frequently than females. No difference could be found in the incidence of venous thrombosis or bleeding complications between the genders.

Cardiovascular risk factors (III)

Sixty-three out of 132 patients (48 %) had one or more vascular risk factors, and 69 patients (52 %) had no vascular risk factors (Table 11). Eleven per cent of the patients (n = 14) had several vascular risk factors. At diagnosis the patients without vascular risk factors had higher platelet counts (median 943 x 10^9/, range 496-2175 x 10^9/l) than those with risk factors (median 833 x 10^9/, range 500-1757 x 10^9/l), (p = 0.04), but no difference in the peak platelet counts between the groups could be detected. The patients with one or more vascular risk factors were significantly older than those without risk factors (median age 58 vs. 46 years, respectively). The gender distribution and the proportion of the patients treated with platelet-lowering drugs or ASA did not differ between the groups.

The presence of one or more vascular risk factors increased the risk of arterial thrombotic complications (Table 12). Approximately half of the patients (52 %) with at least one vascular risk factor, but only 29 % of those without risk factors, experienced arterial thrombosis (p = 0.01). In multivariate analysis the only independent risk factor was smoking (p = 0.01); none of the remaining risk factors contributed significantly to arterial thrombotic complications (Table 13). No significant difference in the age distribution between the smokers and non-smokers could be detected. The impact of smoking on the risk of complication was gender-dependent. Among females arterial complications occurred in 60 % (9/15) of the smokers but only in 15 % (12/82) of the non-smokers (p = 0.002), whereas among males no statistically significant difference was seen in arterial complications between smokers and non-smokers (Table 14).

Cardiovascular risk factors did not predict the risk of venous thrombosis.

Table 11. Vascular risk factors in 132 patients with ET.

	No. of patients with risk factor	Age (years) median	range	Sex Female	Male
Smoking	32	53	24-79	15	17
Hypertension	27	56	41-72	16	11
Hypercholesterolaemia	21	59	41-79	12	9
Diabetes mellitus	4	66	61-73	0	4
One or more risk factors	63	58	24-79	33	30
No risk factors	69	46	19-88	37	32

Table 12. Arterial thrombosis in relation to the risk factors in 132 patients with ET.

Risk factor	No. of patients with risk factor	Per cent of patients with thrombosis		
		Risk factor +	Risk factor -	*P*-value
Smoking	32	59%	34%	0.02
Hypertension	27	48%	41%	ns
Hypercholesterolaemia	21	47%	37%	ns
Diabetes mellitus	4	75%	39%	ns
One or more risk factors	63	52%	29%	0.01
Male gender	62	58%	24%	0.0001

Table 13. The significance of vascular risk factors and gender for the risk of arterial thrombosis. Multivariate analysis.

Risk factor	**p-value**
Smoking	0.01
Hypertension	0.34
Hypercholesterolaemia	0.45
Diabetes mellitus	0.15
Male gender	0.0001

Table 14. The effect of smoking on arterial thrombotic complications according to gender.

	Arterial thrombosis		
	smokers	**non-smokers**	**p-value[1)]**
Males	10/17 (59%)	26/45 (58%)	ns
Females	9/15 (60%)	8/55 (15%)	0.002

[1)] Fischer's exact test

***In vitro* colony formation of haematopoietic progenitors (IV)**

The presence of any spontaneous colony formation increased the risk of arterial thrombosis markedly (Table 15). Forty-three per cent of the patients (49/114) with spontaneous colony formation (megakaryocytic and/or erythroid), but only 20 % (8/40) without it, had an arterial thrombosis ($p = 0.02$). Spontaneous colony formation was also more often seen in patients with an arterial thrombotic complication at diagnosis than in those without thrombosis at diagnosis (all patients $p = 0.01$, patients younger than 45 years $p = 0.04$). In the patients younger than 45 years spontaneous megakaryocytic growth alone had prognostic value: 44 % of the patients (15/34) with spontaneous megakaryocytic colony growth and 14 % (3/21) of those without spontaneous colony formation experienced an arterial thrombotic complication during the course of the disease ($p = 0.04$) (Table 15). When all patients were included, erythroid or megakaryocytic

colony formation did not predict arterial thrombosis. In multivariate analysis of all patients the male gender (p = 0.001, 95 % CI 1.75-7.37) and any spontaneous colony formation (p = 0.009, 95% CI 1.40-10.30) remained as independent risk factors for arterial thrombosis. In the patients younger than 45 years also spontaneous megakaryocytic colony formation (p = 0.04) remained as an independent risk factor. Spontaneous colony growth did not predict the risk of venous thrombosis or bleeding complications.

Table 15. The correlation between spontaneous colony formation and the risk of arterial thrombosis in all patients (n = 154) and patients younger than 45 years of age (n = 55).

	Number of patients with arterial thrombosis / total (% with complication)			
Growth pattern	All patients	p	< 45 years	p
Any spontaneous colony growth	49 / 114 (43 %)		18 / 46 (39 %)	
Normal colony growth	8 / 40 (20 %)	0.02	0 / 9 (0 %)	0.04
Spontaneous CFU-Meg growth	38 / 91 (44 %)		15 / 34 (44 %)	
Normal CFU-Meg growth	19 / 63 (30 %)	0.19	3 / 21 (14 %)	0.04
Spontaneous BFU-E growth	38 / 88 (43 %)		13 / 36 (36 %)	
Normal BFU-E growth	19 / 66 (29 %)	0.09	5 / 19 (26 %)	0.67

Platelet function (IV)

Abnormal platelet aggregation did not correlate significantly with the risk of thrombotic or bleeding complications. However, there was a trend towards fewer complications among the patients with abnormal platelet aggregation. Arterial thrombosis was seen in 43 % of the patients with sufficient platelet aggregation and in 24 % of the patients with defective platelet aggregation (p = 0.33). The corresponding figures for venous thrombosis and major bleedings were 21 % vs. 7 % (p = 0.33) and 36 % vs. 12 % (p = 0.12), respectively.

Normal colony formation and abnormal platelet aggregation response showed each a trend towards a decreased risk of arterial thrombotic complications. Only 6 % (1/15) of the patients with both of these favourable findings experienced an arterial thrombosis, while 38 % (15/40) of the remaining patients had arterial complications (p = 0.04).

Outcome of pregnancy in ET (V)

Eighteen of the 40 pregnancies (45 %) of 16 females were complicated. Fifteen pregnancies (38 %) ended in miscarriage; 13 of these occurred during the first trimester comprising 87 % of all miscarriages. Three pregnancies ending in live birth were complicated by eclampsia or pre-eclampsia. Altogether 25 pregnancies (62 %) resulted in live birth of 26 newborns. The clinical course of pregnancy was uneventful in all 11 pregnancies of seven women. In the remaining nine patients 18/29 (62 %) pregnancies were complicated. Five females had more than one complicated pregnancy.

Three out of 26 placentas appeared clinically abnormal. In two pregnancies leading to a stillbirth the placentas showed calcification and multiple infarcts, while one pregnancy with large areas of placental infarctions resulted in a preterm delivery of a small-for-date infant.

The foetal growth was within normal limits in 24 of the 25 pregnancies resulting in live birth with the median length of gestation of 39 weeks. No abnormal bleedings during the delivery were reported. The median birth weight was 3190 g (range 500-4540 g). The platelet count was analysed in 12 newborns (median 284 x 10^9/l, range 192-540 x 10^9/l), and in five of them the platelet count was above the normal limit (> 290 x 10^9/l).

The platelet count before the conception or during the first trimester did not correlate with the outcome of the pregnancy. The median platelet count in 16 pregnancies resulting in live birth was 757 x 10^9/l and in 13 pregnancies ending in pregnancy loss 835 x 10^9/l. Other ET-related symptoms prior to or during the pregnancy were not found to predict the appearance of pregnancy complications.

Impact of treatment for ET on the course of pregnancy

Treatment for ET was or had been given to 11 of the 16 females (67 %) in connection with 13 pregnancies, either during the pregnancy or before conception or both. ASA was given in altogether ten pregnancies of nine patients in daily doses ranging from 50 to 250 mg. Two of these patients were already on ASA treatment before the conception and continued with it throughout the pregnancy. In the other seven patients the treatment was started at 4 - 27 weeks of gestation, in four of them during the first trimester. Five patients were on IFN treatment. Three of them discontinued IFN after conception and switched over to ASA, one patient continued with IFN from week 15 onwards after an eight-week break, and one patient continued with the IFN treatment throughout the pregnancy. Both patients on IFN treatment during the pregnancy also received concomitantly ASA. Two females with three pregnancies had been treated with busulphan two months and 26 months before conception.

Pregnancy-related complications occurred in 18 of the 27 pregnancies (67 %) in females without any treatment, whereas none of the 13 pregnancies in females with treatment before or during the pregnancy were complicated ($p < 0.001$). The live-birth rate in the treated patients (13/13 pregnancies, 100 %) was higher than that in the untreated patients (12/27 pregnancies, 44 %) ($p < 0.001$) (Table 16). Eight out of eight pregnancies (100 %) with ASA alone versus 12 out of 27 pregnancies (44 %) without any treatment resulted in live birth ($p = 0.01$).

Table 16. The impact of treatment on the pregnancy outcome.

	Live birth	Miscarriage
No treatment	12	15
ASA alone	8	0
Platelet lowering treatment ± ASA	5	0

DISCUSSION

For clinicians ET is still both a diagnostic and therapeutic challenge. Of the MPDs the prognosis of ET is the most favourable. It is generally agreed that the life expectation of patients with ET does not markedly differ from that of the age- and sex-matched control population. At the time of the diagnosis patients are often young and symptomless and, due to the wide use of automatic cell counters, thrombocytosis is often a chance finding. According to previous studies, many patients are symptomless for years, but there is a danger of life-threatening and serious complications mostly caused by arterial thrombosis and less often by venous thrombosis or bleedings. The diagnosis of ET as such is not an indication for treatment, and the indications are still controversial. Most previous studies dealing with risk factors have been published in countries, such as those in southern Europe, where the cardiovascular morbidity among the population is generally lower than in Finland. Thus the risk of complications in Finnish ET patients may be different from that in some other populations. The aim of the present study was to describe the diagnostic and prognostic features as well as the outcome of Finnish patients with ET.

Diagnosis of ET

The goal of the diagnosis of thrombocytosis is to distinguish ET from other MPDs and RT. The differential diagnosis of ET from the other MPDs is usually relatively straightforward and based on adequate iron stores, normal red cell count, as well as on the lack of collagen fibrosis and karyotype abnormalities typical of CML. Difficulties usually appear in the differential diagnosis between ET and RT. Reliable diagnostic methods would be of importance, since RT hardly ever causes problems and complications which the patients with ET are prone to. In addition, the platelet lowering treatments used in ET carry a potential of severe side-effects. Therefore positive diagnostic criteria and the evaluation of diagnostic tools are needed.

In the 1980's the PVSG has proposed widely used diagnostic criteria for ET (Murphy et al. 1986). Although the diagnosis of the patients in the present study has been based on the criteria of PVSG with some modifications, this classification has considerable, well-known limitations. The diagnosis of ET is based on the exclusion of other causes of thrombocytosis, and the lower limit of the platelet count justifying the diagnosis of ET, 600 x 10^9/l, is too high. Previous studies,

as well as the present one, show that patients may experience thrombotic complications with platelet counts only slightly above (Hehlmann et al. 1988, Regev et al. 1997, Lengfelder et al. 1998), or even within, the normal range. A recent proposal for revised diagnostic criteria for ET has defined the platelet count of 400 x 10^9/l as the lower diagnostic limit (Michiels and Juvonen 1997). In addition, during the recent years new diagnostic tools have been proposed and their impact is not yet well defined. Positive criteria that might support the diagnosis of ET are bone marrow morphology (Michiels and Thiele 2002, Thiele and Kvasnicka 2005), enlarged spleen, karyotype abnormalities, platelet volume and distribution width, platelet nucleotide ratio (Dudley et al. 1989), abnormal platelet function studies (Sehayek et al. 1988, Zahavi et al. 1991), spontaneous colony formation in *in vitro* cultures of haematopoietic progenitors (Juvonen et al. 1993, Kutti and Wadenvik 1996, Westwood and Pearson 1996, Michiels and Juvonen 1997), and JAK2 mutation (Campbell and Green 2005, Tefferi and Gilliland 2005).

The WHO citeria (Imbert et al. 2001) emphasize histological findings of the bone marrow biopsy, and histology has been proposed as a tool to establish the diagnosis of ET. Clustering of enlarged megakaryocytes with multilobulated nuclei is a common finding in ET, as also shown in the present study. It has even been proposed that bone marrow biopsy could facilitate an earlier diagnosis in patients with only moderately elevated platelet counts (400 – 600 x 10^9/l) (Lengfelder et al. 1998, Sacchi et al. 2000). The evaluation of bone marrow morphology has been proposed as a predictor of a disease subtype, indicating that “true ET” may be distinguished form early myelofibrosis or latent PV (Thiele et al. 2000, Thiele and Kvasnicka 2005). The prognostic significance of bone marrow findings has been shown in one study (Annaloro et al. 1999). The diagnostic classification based on bone marrow histology is, however, limited by the fact that megakaryocyte morphology and reticulin grading are difficult to assess in a reproducible manner (Harrison 2005b).

The presence of spontaneous colony formation of haematopoietic progenitors has been included as a positive marker in the less well known European Clinical and Pathological criteria (Michiels and Juvonen 1997), but not in the PVSG or WHO criteria. In the three largest studies, 63-100 % of the patients with ET have shown spontaneous megakaryocytic colony formation and 59-88 % spontaneous erythroid colony formation (Juvonen et al. 1993, Florensa et al. 1995, Rolovic et al. 1995). Our study confirmed the findings of a previous study of the present authors with a smaller patient group (Juvonen et al. 1993) that either megakaryocytic or erythroid spontaneous colony

formation is seen in the majority (74 %) of the ET patients. The majority of the patients in the present study also showed deficient platelet aggregation (75 %). Of the 55 patients tested for both colony formation and platelet aggregation 51 had abnormal results in both or at least one of these tests. It is therefore obvious that abnormal *in vitro* cultures and platelet function studies support the diagnosis of MPD. These tests are, however, technically demanding, difficult to standardize, and available only in relatively few haematological laboratories. The differential diagnostic usefulness of platelet function studies may be limited by an overlap between myeloproliferative and reactive thrombocytosis, as seen in some previous studies (Sehayek et al. 1988, Majer et al. 1991).

The over-expression of the PRV-1 gene, which has been a molecular marker in PV, can be detected in approximately half of the patients with ET. However, as a diagnostic tool it has turned out to be a disappointment, because the PRV-1 over-expression is constitutively detectable in bone marrow cells and it does not differentiate PV or ET from reactive disorders or from other MPDs (Bock et al. 2003, Tefferi et al. 2003, Passamonti et al. 2004a). It is obvious that in the future the $JAK2^{V617F}$ mutation screening will be included in the diagnostic algoritm (Campbell and Green 2005, Tefferi and Gilliland 2005), as it offers a diagnostic tool for a MPD in half of the ET patients. The precise role of the JAK2 mutation remains to be seen. The present studies were performed before the JAK2 era, and the JAK2 mutation data is not available. Thus, the relationship between the JAK2 mutational status and the classical diagnostic findings in ET could not be evaluated.

The proportion of our patients whose diagnosis was made by chance was higher than that in several earlier studies (Hehlmann et al. 1988, Cortelazzo et al. 1990, Fenaux et al. 1990, Randi et al. 1991, van Genderen et al. 1997a, Lengfelder et al. 1998). It was interesting that in all age groups the disease was diagnosed in a significantly higher proportion of females by chance, and the peak incidence of the disease among the female patients was two decades earlier than that in the males (40-50 years vs. 60-70 years, respectively). The active use of health care services by females in general together with systematic maternity care may at least partly explain the differences in the distribution of age and symptoms between the sexes, and the high incidence of atherosclerosis in the Finnish male population (Uemura and Pisa 1988) may contribute to these findings.

Thrombohaemorrhagic complications and prognostic factors

The precise incidence of thrombohaemorrhagic complications in ET is difficult to ascertain, since most studies are retrospective by nature, event definitions and result reporting vary, and there may have been a bias with the patient selection. In previous large studies the incidence of major thrombotic complications at the diagnosis of ET has varied from 11 to 25 % and during follow-up from 10 to 23 % (Bellucci et al. 1986, Cortelazzo et al. 1990, Fenaux et al. 1990, Colombi et al. 1991, Besses et al. 1999, Jensen et al. 2000a). In the study of Bazzan et al. (1999) with 187 patients the incidence of thrombotic complications during the follow-up of nine years was as high as 50 %, but the patients were originally selected to the special thrombosis unit due to a thrombotic event causing a bias towards a high-risk patient population. In the prospective trial with hydroxyurea (Cortelazzo et al. 1995, Finazzi et al. 2000) the total incidence of thrombosis in the control group comprising high-risk patients was 45 %, while in a prospective study with low-risk patients the incidence was only 7.7 % (Ruggeri et al. 1998). The total rate of thrombotic complications (45 %), especially arterial events, was clearly higher in the Finnish patients with ET than in most of the previous retrospective studies (Bellucci et al. 1986, Chistolini et al. 1990, Cortelazzo et al. 1990, Fenaux et al. 1990, Colombi et al. 1991, van Genderen et al. 1997a, Besses et al. 1999, Jensen et al. 2000a). This may reflect the relatively high incidence of cardiovascular problems in the Finnish population in general (Uemura and Pisa 1988). Indeed, half of our patients had at least one cardiovascular risk factor. The patients of the present study were referred to the participating municipal or central hospitals directly from the primary health care centers, indicating that this patient population may be considered the least selected among the published reports, without any obvious selection bias. Contrary to thrombotic complications, the incidence of bleedings in the present patients was in line with that of the previous reports. The incidence of major bleeding rates varied from 3.6 to 37 % in the previous studies (Bellucci et al. 1986, Cortelazzo et al. 1990, Fenaux et al. 1990, Colombi et al. 1991, Besses et al. 1999, Jensen et al. 2000a) being 21 % in our study.

In the present study the platelet count did not predict thrombotic or bleeding complications, which is in agreement with most of the previous studies as regards to thrombosis (Cortelazzo et al. 1990, Colombi et al. 1991, Bazzan et al. 1999, Besses et al. 1999), but not with the studies showing an increased frequency of bleeding complications at high platelet counts (Bellucci et al. 1986, Fenaux et al. 1990). Age has been associated with an increased risk of thrombosis in four

reports (Cortelazzo et al. 1990, Randi et al. 1992, Besses et al. 1999, Shih et al. 2002). However, in the present study the incidence of thrombotic complications was similar in all age groups, and serious complications occurred also in young and middle-aged patients. The present finding of a higher incidence of major bleedings in older patients has been previously described once (Shih et al. 2002) and it may be associated with the presence of other illnessess and treatments, but no correlation to the use of ASA could be detected.

Previous reports of the influence of cardiovascular risk factors, such as smoking, hypertension, hypercholesterolaemia, and diabetes, on thrombotic complications in ET have been conflicting. In a retrospective study with 148 ET patients hypercholesterolaemia alone was shown to be an independent risk factor (Besses et al. 1999). Watson and Key (1993) reported a study of 46 patients with a follow-up of 50 months, in which vascular risk factors, especially smoking, more than doubled the risk of complications. In the prospective study of Ruggeri et al. (1998) of 65 low-risk ET patients aged less than 60 years, obesity, but no other cardiovascular risk factors, predicted the risk of thrombosis. However, other large studies failed to reveal these associations (Hehlmann et al. 1988, Cortelazzo et al. 1990, Fenaux et al. 1990, van Genderen et al. 1997b, Bazzan et al. 1999, Jensen et al. 2000a). In the present study the median follow-up was long, more than five years, and particularly the results of the predictive value of smoking are in line with those in the study of Watson and Key (1993) with a long follow-up. Smoking favours thrombosis by impairing the endothelial cell function, increasing the concentrations of fibrinogen and vascular cell adhesion molecule-1, altering platelet activation, and decreasing the concentration of high-density lipoprotein cholesterol (Garrison et al. 1978, Reinders et al. 1986, Nowak et al. 1987, FitzGerald et al. 1988, Celermajer et al. 1993, Celermajer et al. 1994, Casey et al. 2004, Cavusoglu et al. 2004). The observation that smoking had a strong predictive value for the development of arterial thrombotic complications in females, whereas there was no correlation between smoking and arterial complications in males, is intriguing. Hormonal differences between the sexes may explain this finding to some extent. Estrogen can prevent cardiovascular complications by several mechanisms, including an increase in the endothelium-dependent vasodilatation and basal arterial diameter and a decrease in the basal vascular resistance, leading to an improvement of the endothelial function (Celermajer et al. 1993, Lieberman et al. 1994, Reis et al. 1994). Estrogen therapy has also been shown to lower LDL cholesterol and raise HDL cholesterol, which improves endothelial function (Miller et al. 1995).

The present finding showing no difference in the arterial thrombotic complications between smoking and non-smoking males remains unexplained.

Knowledge of the prognostic value of spontaneous *in vitro* colony formation of haematopoietic progenitors is limited. In the previous studies with smaller patient populations erythroid spontaneous colony formation did not predict thrombotic complications (Ciaudo et al. 1998, Chiusolo et al. 2001), which is in accordance with the present larger study. The correlation between megakaryocytic spontaneous colony formation and an increased risk of thrombohaemorrhagic complications was already shown in a previous study of 61 patients by the present authors (Juvonen et al. 1993). The present study confirmed this finding in younger patients. The prognostic difference between the megakaryocytic or erythroid colony formation is not clear but it may be only a matter of sample size. In the present study the patients with spontaneous erythroid colony formation showed a trend toward an increased risk of arterial thrombotic complications, though the difference did not reach statistical significance.

Transformation

Myeloproliferative disorders can transform into another disease of the same group, a myelodysplastic syndrome or acute myeloid leukaemia. The rate of fibrotic transformation in the present study was low corresponding to previous large studies (Bellucci et al. 1986, Fenaux et al. 1990, Cervantes et al. 2002). The evolution of ET to PV has previously been described infrequently. In the present study the development of erythrocytosis in 6.5 % of the patients with typical ET was rather unexpected and reflects the close relationship between these disorders. Spontaneous erythroid colony formation was seen in all patients who later developed erythrocytosis but only in 60 % of those with stable ET. Thus, spontaneous erythroid colony formation is not predictive of the development of erythrocytosis in individual patients with newly diagnosed ET, but normal erythroid colony growth may indicate a stable ET.

The rate of myelodysplastic or leukaemic transformation was low (one out of 170 patients), which confirms the opinion that leukaemic transformation is sporadic in untreated patients (Barbui 2004a). The present patient who developed myelodysplastic transformation had earlier been treated with radiophosphorus. None of the patients receiving hydroxyurea showed

leukaemic transformation, but the median follow-up time of five years is relatively short for leukaemic transformation.

Outcome and management of pregnancy

Up till now the published number of pregnancies in patients with ET is limited, and most reports include a small number of patients which may cause a selection bias toward complicated cases. Only a few large reports are, so far, available (Beressi et al. 1995, Cincotta et al. 2000, Wright and Tefferi 2001). The high rate of miscarriages in the present study is contrary to several reports with successful pregnancy outcomes (Jones et al. 1988, Linares et al. 1988, Beard et al. 1991, Chow et al. 1992, Randi et al. 1994, Randi et al. 2000), but in line with the miscarriage rate of 33-40 % in some previous studies (Pagliaro et al. 1996, Bangerter et al. 2000, Cincotta et al. 2000). In the largest report from the Mayo Clinic the rate of miscarriages was as high as 49 % and the rate of the first-trimester abortions 37 % (Wright and Tefferi 2001). The miscarriage rate in ET is clearly higher than that of 12-15 % in the normal population (Houwert-de Jong et al. 1989, Cramer and Wise 2000).

The prediction of the pregnancy-related complications is difficult, as shown in the present study. Neither the platelet count prior to or during the pregnancy nor ET-related symptoms correlate with the pregnancy outcome. In our patient population five out of nine patients with a previous pregnancy complication also had problems during the following pregnancies. Thus, a complication in a previous pregnancy seems to be associated with a tendency to a new complication in the subsequent pregnancies.

The precise pathophysiology of pregnancy complications in ET remains unclear. It has been suggested that normal physiologic changes in pregnancy favouring thrombosis are exacerbated when some other thrombophilic disorder is present. In normal pregnancy increased platelet activation and increased production of TXA_2 are present (Fizgerald et al. 1987). In ET placental insufficiency obviously plays a key role in pregnancy complications (Harrison 2005 a). Several cases of placental thrombosis leading to intrauterine foetal death have been described in the literature (Falconer et al. 1987, Mercer et al. 1988). There may be a direct placental damage, or a shift of prostaglandin/thromboxane ratio may lead to a decreased placental perfusion. Excessive TXA_2 biosynthesis has been described in ET (Zahavi et al. 1991, Rocca et al. 1995). ASA inhibits

TXA_2 and shifts the balance of prostaglandin and thromboxane to antiaggregation, which provides a rational basis for the treatment with ASA during pregnancy in patients with ET. In a recent literature review the live birth ratio among the patients treated with ASA alone or in combination was 68 % but in the untreated patients only 48 % (Griesshammer et al. 2003). The use of ASA was advantageous in some previous studies (Bangerter et al. 2000, Cincotta et al. 2000, Randi et al. 2000) as well as in the present study, but the largest reports from the Mayo Clinic have failed to reveal the benefit of the ASA treatment (Beressi et al. 1995, Wright and Tefferi 2001).

The management issues of ET– whom and how to treat?

All patients with a history of a thrombohaemorrhagic complication or aged higher than 60 years are regarded as high-risk patients, and platelet lowering treatment is indicated in these patients (Cortelazzo et al. 1995). Finnish male patients may be regarded as high-risk patients and therefore platelet lowering treatment should also be actively considered in them. A high platelet count of 1000–1500 x 10^9/l indicates platelet lowering treatment due to an increased risk of bleeding complications (Barbui et al. 2004b, Finazzi and Harrison 2005). Smoking should be discouraged and stopped when the diagnosis of ET is established. In young patients the presence of spontaneous megakaryocyte colony formation may indicate a need of therapy. On the other hand, non-smoking female patients and young patients without any spontaneous megakaryocyte colony formation may be observed without therapeutic interventions.

Some previous reports (Regev et al. 1997, Storen and Tefferi 2001) as well as the present study suggest that if platelet lowering treatment is indicated, the platelet count should be brought back to the normal levels (< 400 x 10^9/l). In younger patients IFN is an effective and safe alternative, but its use is limited by several side-effects. As a single agent hydroxyurea obviously carries only a minimal leukaemogenic risk and is therefore useful in patients not tolerating IFN. In elderly patients (> 70 years) radioactive phosphorus and busulphan are alternative treatments. In the PT1-study, which compared anagrelide and ASA with hydroxyurea and ASA, arterial thrombosis, major bleeding, and myelofibrosis were significantly more frequent in patients treated with anagrelide (Harrison et al. 2005). Thus, the role of anagrelide needs further evaluation.

The efficacy of ASA in the primary prevention of thrombotic complications has recently been shown in PV (Landolfi et al. 2004). Due to the generally increased thromboxane biosynthesis in MPDs, it may be assumed that also patients with ET benefit from low-dose ASA as primary prevention of thrombotic complications; therefore the use of low-dose ASA may be indicated in all ET patients. Among patients with any contraindications to ASA, such as a history of bleeding or high platelet counts (> 1000–1500 x 10^9/l), the need for platelet lowering treatment should be considered. Practical suggestions for the management of ET are presented in Table 17.

In pregnancies of ET patients it seems reasonable to give low-dose ASA since the early weeks of pregnancy or since the pregnancy is being planned. If the patient has a contraindication to ASA and she has experienced a thrombohaemorrhagic complication during her previous pregnancy, platelet lowering treatment with IFN is indicated.

Table 17. Practical suggestions for the management of patients with ET based on the present findings and on the literature.

Risk category	Proposed management	
	ASA	Platelet lowering treatment
Low / intermediate risk	+ / -	-
All of the following:		
Platelet count < 1000–1500 x 10^9/l[1]		
Age < 60 years[1,2]		
Female gender[2]		
No symptoms[1]		
No cardiovascular risk factor(s)[1,2]		
High risk		
Any of the following:		
Male gender[2]	+	+
Platelet count > 1000–1500 x 10^9/l[1]	-	+
Age > 60 years[1,2]	+	+
Symptoms[1]	+	+
Prior thrombosis[1]	+	+
Prior haemorrhage[1]	-	+
Cardiovascular risk factor(s)[1,2]	+	+

[1]literature
[2]present findings

SUMMARY AND CONCLUSIONS

The aims of the present study were to evaluate the diagnostic findings, clinical course, and prognostic factors of essential thrombocythaemia in 170 Finnish patients with ET diagnosed and followed during the years 1980-1995.

At the time of the diagnosis the median age of the patients was 52 years with a wide range from 19 to 88 years. One third of the patients were younger than 45 years. There was a slight excess of female patients with an F : M ratio of 1.3 : 1. The peak incidence in females was two decades earlier than in males (40-49 years vs. 60-69 years, respectively). In the majority of the patients ET was diagnosed by chance, and females were more frequently asymptomatic at the time of the diagnosis than males. In the symptomatic patients the most frequent cause for diagnostic evaluations was a thrombotic complication. Of the thrombotic complications nearly half were major disturbances of the cerebral circulation. About two thirds of the patients presented with a platelet count of $\leq 1000 \times 10^9$/l. The most common positive findings supporting the diagnosis of ET, in addition to conventional criteria, were an increased number of megakaryocytes with abnormal morphology in a bone marrow aspirate, platelet aggregation defects in the platelet function studies, and the presence of spontaneous erythroid and/or megakaryocytic colony formation in the *in vitro* cultures of haematopoietic progenitors. Approximately 70 % of the patients had either spontaneous megakaryocytic or erythroid colony formation, while about 30 % of the patients showed a normal haematopoietic progenitor growth pattern.

At diagnosis or during the course of the disease only about 20 % of the patients remained asymptomatic. Approximately half of the patients experienced a major thrombohaemorrhagic complication. Thrombotic complications were more frequent than bleedings (45 % of the patients). Microvascular symptoms were experienced by half of the patients. About 20 % of the patients had major bleeding complications.

Transformation to myelofibrosis was a rare event in the present ET patients, but 6.5 % of the patients developed erythrocytosis during the follow-up. No leukaemic transformation occurred.

The proportion of the patients with arterial thrombotic events was higher in the Finnish patients compared to the findings of most reports dealing with other populations. The platelet count did not predict thrombotic or bleeding complications. Age over 60 years increased the risk of major bleedings, but, in the present Finnish patients the occurrence of thrombotic complications was similar in all age groups, unlike in some other reports. This study showed, for the first time, male gender as a risk factor for thrombosis in ET. Smoking, especially in females, correlated with a risk of thrombosis. The presence of any spontaneous colony formation increased the risk of arterial thrombosis in all patients, while in young patients also spontaneous megakaryocytic colony formation had predictive value.

Pregnancies in patients with ET carried an increased risk of complications. Eighteen out of 40 pregnancies (45 %) were complicated. Fifteen pregnancies (38 %) resulted in stillbirth, most of them during the first trimester. A complication during a previous pregnancy predicted problems also during subsequent pregnancies. Contrary to a previous large report, treatment with ASA alone or in combination with platelet lowering drugs significantly improved the outcome of the pregnancy in the present retrospective analysis.

Conclusions

The main findings of the present study were the following: Spontaneous colony formation in the *in vitro* cultures of haematopoietic progenitors supported the diagnosis in a considerable proportion of the patients with ET. The presence of any spontaneous colony formation in all patients and the presence of spontaneous megakaryocytic colony growth in the patients younger than 45 years had also prognostic value. Male gender and smoking in female patients increased the risk of thrombotic complications. The occurrence of thrombotic complications in females was less frequent than in males, but the risk of pregnancy complications was considerable.

The conclusions of the treatments and their effects are limited due to the retrospective nature of the study, heterogeneous treatments, and the long period covered, but some clearly suggestive observations were made, including the benefit of ASA particularly during pregnancies.

In the future the present findings of risk factors in ET as well as treatment outcome in the pregnancies of ET patients should be taken into consideration when planning treatment strategies for Finnish patients, including the indications for low-dose ASA or platelet lowering drugs and the importance of effective interventions to other cardiovascular risk factors.

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