MULTIDISCIPLINARY APPROACH IN THE DIAGNOSIS AND MANAGEMENT OF PHILADELPHIA CHROMOSOME-NEGATIVE MYELOPROLIFERATIVE NEOPLASMS

Ph.D. dissertation

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LIST OF ABBREVIATIONS

2D	two-dimensional
3D	three-dimensional
3DSTE	three-dimensional speckle-tracking echocardiography
AAEF	active atrial emptying fraction
AASV	active atrial stroke volume
AEC	absolute eosinophil count
AMI	acute myocardial infarction
AML	acute myeloid leukaemia
AP2CH	apical two-chamber (view)
AP4CH	apical four-chamber (view)
ASA	acetylsalicylic acid
ASM	aggressive systemic mastocytosis
B-ALL	B-cell acute lymphoblastic leukaemia
CEL/HES	chronic eosinophilic leukaemia/hypereosinophilic syndrome
CEL-NOS	chronic eosinophilic leukaemia – not otherwise specified
CM	cutaneous mastocytosis
CML	chronic myeloid leukaemia
CNS	central nervous system
CS	circumferential strain
CV	cardiovascular
DM	diabetes mellitus
DVT	deep vein thrombosis
ECLAP	European Collaboration Study on Low-dose Aspirin in Polycythemia
ECNM	European Competence Network on Mastocytosis
EDTA	ethylenediaminetetraacetic acid
EDV	end-diastolic volume
est LV MASS	estimated left ventricular mass
ESV	end-systolic volume
ET	essential thrombocythaemia
EF	ejection fraction

F	female
FFPE	formaldehyde-fixed and paraffin-embedded
FGFR1	fibroblast growth factor receptor 1
FIP1L1	Fip1-like-1
FISH	fluorescence *in situ* hybridization
Hb	haemoglobin
Hct	haematocrit
HES	hypereosinophilic syndrome
HSC	haematopoietic stem cell
ICD	International Classification of Diseases
IFN-alpha	interferon-alpha
iHES	idiopathic hypereosinophilic syndrome
ISM	indolent systemic mastocytosis
JAK2	Janus kinase 2
LA	left atrial
LAD	left anterior descending (coronary artery)
LCX	left circumflex (coronary artery)
LIMA	left internal mammary artery
LM	left main (coronary artery)
LS	longitudinal strain
LV	left ventricle / left ventricular
M	male
MCA	middle cerebral artery
MCD	mast cell disease
MCL	mast cell leukaemia
MCS	mast cell sarcoma
MDS	myelodysplastic syndrome
MF	myelofibrosis
MM	multiple myeloma
MPD	myeloproliferative disease / disorder
MPN	myeloproliferative neoplasm
NSTEMI	non–ST-segment elevation myocardial infarction
PAD	peripheral arterial disease

PAEF	passive atrial emptying fraction
PASV	passive atrial stroke volume
PCA	posterior cerebral artery
PCI	percutaneous coronary intervention
PCR	polymerase chain reaction
PDGFRA /B	platelet-derived growth factor receptor alpha/beta
PE	pulmonary embolism
Ph	Philadelphia chromosome
PLT	platelet
PMF	primary myelofibrosis
PTCL	peripheral T-cell lymphoma
PV	polycythaemia vera
PVSG	Polycythemia Vera Study Group
RBC	red blood cell
RC	right coronary (artery)
RS	radial strain
RT-PCR	real-time polymerase chain reaction
SM	systemic mastocytosis
SM-AHNMD	systemic mastocytosis with an associated clonal haematological non-mast cell lineage disease
SV	stroke volume
SVG	saphenous vein graft
TAEF	total atrial emptying fraction
TASV	total atrial stroke volume
TIA	transient ischaemic attack
TTE	transthoracal echocardiography
UP	urticaria pigmentosa
VBI	vertebrobasilar insufficiency
V_{max}	maximum (left atrial) volume
V_{min}	minimum (left atrial) volume
V_{preA}	(left atrial) volume before atrial contraction
WBC	white blood cell
WHO	World Health Organization

TABLE OF CONTENTS

1 INTRODUCTION

Myeloproliferative diseases (MPDs) or neoplasms (MPNs) represent a heterogeneous group of clonal haematopoietic stem cell (HSC) disorders characterized phenotypically by an abnormal accumulation of mature-appearing myeloid cells of one or more lineages [1]. The so-called "classic" MPDs – now referred to as "classic" MPNs – include chronic myeloid leukaemia (CML), polycythaemia vera (PV), essential thrombocythaemia (ET), and primary myelofibrosis (PMF) [2]. The Polycythaemia Vera Study Group (PVSG) and the World Health Organization (WHO) classifications distinguish the Philadelphia chromosome (Ph)-positive CML from the Ph-negative entities ET, PV, and PMF [3].

Following the discovery of the disease-causing *BCR–ABL1* mutation in CML, a number of other fusion genes and oncogenic tyrosine kinase mutations such as Fip1-like-1–platelet-derived growth factor receptor alpha gene fusion (*FIP1L1–PDGFRA*) as well as Kit (*KIT D816V*) and Janus kinase 2 (*JAK2 V617F*) mutations have also been identified [4]. The 2008 WHO classification of myeloid neoplasms and acute leukaemia incorporating this new knowledge provides a novel, morphology-, cytogenetics-, and molecular diagnostics-based nomenclature and classification of MPDs [5].

In this revised classification, the expression "myeloproliferative disorder" has been replaced by "myeloproliferative neoplasm". In addition to changes in the nomenclature, a significant modification was – based on their shared features – the enrolment of mast cell diseases (MCDs) among MPNs and the re-organization of chronic eosinophilic leukaemia/hypereosinophilic syndrome (CEL/HES) into "CEL, not otherwise specified (CEL-NOS)" as well as "myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of PDGFRA, platelet-derived growth factor receptor beta (PDGFRB), or fibroblast growth factor receptor 1 (FGFR1)" (Table 1) [5].

Eight clinicopathological entities fall therefore currently under the category of MPNs: *BCR–ABL1*-positive CML and seven *BCR–ABL1*-negative conditions including chronic neutrophilic leukaemia, PV, PMF, ET, CEL-NOS, mastocytosis, and MPNs – unclassifiable [6]. The term "Ph-negative MPNs" is still widely used as a synonym for classic *BCR–ABL1*-negative MPNs (PV, ET, PMF) and, in the broader sense, also for other *BCRABL1*-negative myeloproliferative conditions such as certain rare entities like HES or systemic mastocytosis (SM).

Table 1. *Classification of myeloid neoplasms according to the 2008 WHO scheme [5]*

1. Myeloproliferative neoplasms (MPNs)
1.1. Chronic myeloid leukaemia (CML), *BCR–ABL1*-positive
1.2. Polycythaemia vera (PV)
1.3. Essential thrombocythaemia (ET)
1.4. Primary myelofibrosis (PMF)
1.5. Chronic neutrophilic leukaemia (CNL)
1.6. Chronic eosinophilic leukaemia, not otherwise specified (CEL-NOS)
1.7. Mast cell disease (MCD)
1.8. MPN, unclassifiable
2. Myeloid and lymphoid neoplasms associated with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1*
3. Myelodysplastic/myeloproliferative neoplasms (MDSs/MPNs)
3.1. Chronic myelomonocytic leukaemia (CMML)
3.2. Juvenile myelomonocytic leukaemia (JMML)
3.3. Atypical chronic myeloid leukaemia, *BCR–ABL1*-negative (aCML)
3.4. MDS/MPN, unclassifiable
4. Myelodysplastic syndromes (MDSs)
5. Acute myeloid leukaemia (AML)

FGFR1, fibroblast growth factor receptor 1; PDGFRA, platelet-derived growth factor receptor alpha; PDGFRB, platelet-derived growth factor receptor beta.

Polycythaemia vera (PV) is the most common entity among all Ph-negative myeloproliferative disorders which, due to its vascular complications, represents an interdisciplinary significance. PV is characterized by the trilineage clonal proliferation of HSC-derived haematopoietic progenitors resulting in the expansion of the erythrocyte mass. In addition to blood hyperviscosity, the increased red blood cell (RBC) mass results in a higher risk for thrombosis, poor quality of life, and a shorter life expectancy. PV warrants particular attention for the higher risk of cardiovascular (CV) and cerebrovascular events as leading determinants of morbidity and mortality. Predominant are arterial thrombotic events, in particular large vessel arterial events including cerebrovascular events, myocardial infarction, and peripheral arterial occlusion. PV-related haemostatic abnormalities due to qualitative disorders of erythrocytes, neutrophils, and platelets (PLTs) and the pathogenesis of the major thrombotic complications are extensively investigated yet not fully unravelled. From clinical and therapeutical aspects, the role and significance of additional risk factors in the development of PV-associated thrombotic events is of major importance – over the last few years, this topic has been actively investigated.

Systemic mastocytosis (SM), an exceedingly rare of Ph-negative MPNs is considered as an orphan disease with less known clinical presentation, prognosis, and challenging treatment. The multidisciplinary significance of SM can be attributed to the pathological accumulation of morphologically and immunophenotypically abnormal mast cells in one or more organ systems. From mast cell diseases (MCDs) our research focussed on systemic mastocytosis (SM) due to its low incidence rate, heterogeneous manifestation, and clinical complexity and the diagnostic difficulties often associated with it. As the presentation of SM

may vary from asymptomatic to severe forms, its diagnosis can be especially challenging, both from a clinical and pathological perspective. Up to now, only very limited epidemiological data are available on SM. Although a small set of regional data have been collected through the European Competence Network on Mastocytosis (ECNM), no Hungarian data at all are present.

Among Ph-negative MPNs, hypereosinophilic syndromes (HESs) make up the most heterogeneous and widely debated group of diseases. The clinical presentation of HES is highly variable ranging from a relatively asymptomatic disease to endomyocardial fibrosis. Although HES may also be associated with other organ system failures, cardiac involvement and in particular Loeffler's endocarditis still remain its best known manifestation. As HES represents a very heterogeneous group of diseases, its definition has been strongly debated for decades. Thanks to the current molecular and immunological diagnostic methods, an aetiology-based classification in certain types of HESs is now possible, yet at the price of an even more complicated terminology.

The clinical presentation of HES is highly variable ranging from a relatively asymptomatic disease to endomyocardial fibrosis. A well-known and frequent cardiac manifestation of HES is Loeffler's endocarditis [7-9]. The cardiac involvement begins with eosinophilic infiltration, followed by an intermediate thrombotic stage, and finally evolving into a late fibrotic stage [7]. Enlarged atrium with normal-sized left ventricle (LV) is a minor criterion for endomyocardial fibrosis. At this moment, little is known about left atrial (LA) function in HES. In our research, we investigated a) the ability of a novel non-invasive clinical tool (three-dimensional speckle tracking echocardiography, 3DSTE) to reveal any change in the cardiac functional parameters in a sub-type of HES considered idiopathic in its etiology HES (iHES) without manifest organ damage (as determined by conventional diagnostic methods) as well as b) the left ventricular (LV) rotational mechanics in clinically symptomatic Loeffler's endocarditis.

This work discusses the clinical, laboratory, and molecular characteristics of selected Ph-negative MPNs like PV, SM, and HES and examines how heterogeneous their clinical appearance can be despite their common HSC-derived origin. The diagnosis and treatment of all three Ph-negative MPNs require a strong multidisciplinary (cardiological – neurological – dermatological) approach and a close co-operation with other clinical specialists.

2 AIMS

The aim of our research was to create a retrospective database of Ph-negative MPNs including three separate cohorts of patients diagnosed with PV, SM, or HES. This work

evaluates and analyses separately the PV, SM, and HES patient cohorts of the 2nd Department of Internal Medicine and Cardiology Centre, Albert Szent-Györgyi Health Centre, Faculty of Medicine, University of Szeged, with the following objectives in each cohort:

I. PV cohort:

Given that PV is the most common type of Ph-negative MPNs and in this group of diseases, vascular events are of outstanding significance and diagnostics, efficacious treatment can only be achieved on a multidisciplinary level, we assessed our patient population in the following aspects:

a) to evaluate the incidence of thrombotic events prior to and during follow-up; and to investigate the main clinical characterteristics of PV patients, either with or without thrombotic complications in the follow-up period;

b) to assess the major cerebrovascular and cardiac thrombotic complications as the most serious thrombotic complications in the PV cohort; and to investigate whether any typical neurological or cardiac lesion(s) could be identified which might be specific to, or characteristic of PV; and

c) to evaluate the contribution of the main CV risk factors present at time of haematological diagnosis of PV as possible additional risk factors for subsequent thrombotic complications.

II. SM cohort:

Analyses in our SM patient group were driven by the orphan nature and the diagnostic, therapeutic, and prognostic difficulties usually associated with this condition as follows:

a) to describe the clinicopathological findings along with bone marrow histological features, molecular characteristics, and laboratory parameters at presentation in a large cohort of SM patients;

b) to evaluate the frequency of *KIT D816V* mutation in SM subgroups;

c) to estimate the life expectancy of SM patients compared to age- and sex-matched controls; and to evaluate the prognostic relevance of the WHO classification of SM in the investigated patient population; and

d) to quantify the cumulative incidence of SM in the South Great Plain region of Hungary.

III. HES cohort:

HES, a condition with highly variable clinical presentation and organ involvement also belongs to the group of rare Ph-negative MPNs. In this patient population the aims of our

investigations were:

a) to compare LA volumetric, volume-based functional, and strain parameters obtained by three-dimensional speckle-tracking echocardiography (3DSTE), a novel, non-invasive clinical tool for volumetric and strain analysis, between HES patients and matched controls; and

b) to demonstrate LV rotational mechanics in a unique case with Loeffler's endocarditis.

3 PATIENTS, METHODS, AND RESULTS

As basis for this research, we retrospectively established a database for scientific research, focussing especially on PV, SM, and HES cases diagnosed at the 2nd Department of Internal Medicine and Cardiology Centre between 1998 and 2014 (Table 2).

Table 2. *Overview of the main characteristics of the investigated study population*

Main characteristics	*Patient population*		
	PV	***SM***	***HES***
Data collection period	*1998–2014*	*2001–2013*	*2001–2014*
Total number of patients	**108**	**35**	**11**
Males [N (%)]	57 (52.8%)	19 (54.2%)	8 (72.7%)
Females [N (%)]	51 (47.2%)	16 (45.7%)	3 (27.2%)
Median age at diagnosis (years) (range)	62.6 (24.8–82.0)	57 (31–85)	59 (33–77)
Median follow-up (months) (range)	54 (0.0–16.1)	30.5 (1–240)	45 (2–168)

HES, hypereosinophilic syndrome; PV, polycythaemia vera; SM, systemic mastocytosis.

Our investigations were conducted with the approval of the Regional and Institutional Human Medical Biological Research Ethics Committee of the Albert Szent-Györgyi Health Centre, University of Szeged and in accordance with the Declaration of Helsinki principles. Written informed consent was not required from the subjects. The review of trial subjects' relevant medical data was done by using MedSolution, the healthcare data management system of the Albert Szent-Györgyi Health Centre, Faculty of Medicine, University of Szeged.

3.1 POLYCYTHAEMIA VERA

3.1.1 GENERAL CONSIDERATIONS AND BACKGROUND OF THE POLYCYTHAEMIA VERA STUDY

PV is generally characterized by erythrocytosis, but other signs and symptoms like leukocytosis, thrombocytosis, splenomegaly, vasomotor disturbances, thrombosis, bleeding,

or pruritus may also be present. The incidence of PV ranges in European Union from 0.4 to 2.8 cases per 100,000 persons per year [10]. PV occurs in all populations and in all age groups including young adults and occasionally children and adolescents, too. The median age at diagnosis was around 61 years (range: 18–95 years) in a group of 1545 patients with WHO-defined PV assessed by the International Working Group for Myeloproliferative Neoplasms Research and Treatment [11]. The incidence of PV was slightly higher in men than in women (2.8 vs. 1.3 cases per 100,000 persons per year), with the highest rates in men aged 70–79 years (24 cases per 100,000 persons per year) [12].

The diagnosis of PV is based on the current WHO criteria including clinical and laboratory findings and the molecular analysis of *JAK V617F* mutation [13]. The diagnosis is established if both major criteria and at least one minor criterion, or the first major criterion and at least two minor criteria are present (Table 3).

Table 3. *Diagnostic criteria of polycythaemia vera [13]*

Major criteria
1. Hb >18.5 g/dL in men, 16.5 g/dL in women, or other evidence of increased red cell volume
2. Presence of *JAK2 V617F* or other functionally similar mutation such as *JAK2* exon 12 mutation
Minor criteria
1. Bone marrow biopsy showing hypercellularity for age with trilineage growth (panmyelosis) with prominent erythroid, granulocytic, and megakaryocytic proliferation
2. Serum erythropoietin level below the reference range for normal
3. Endogenous erythroid colony formation *in vitro*

Hb, haemoglobin; JAK2, Janus kinase 2.

JAK2 V617F is by far the most prevalent mutation in *BCR–ABL1*-negative MPNs: it occurs in ~95% of patients with PV, in ~55% with ET, and in ~65% with PMF, respectively [6].

The Janus kinase/signal transducer and activator of transcription (JAK-STAT) signalling pathway is of central importance in a number of cellular processes including proliferation, survival, and normal functioning of haematopoietic and other cells [6]. The vast majority (~96%) of PV patients have a *JAK2 V617F* somatic activating mutation in exon 14 while the rest of them (~3%) exhibit a *JAK2* exon 12 mutation [14]. The overall median survival in PV is 14 years with a median survival time of 24 years for patients younger than 60 years [15]. The 10-year risk is 3% and 10% for leukaemic transformation and fibrotic transformation, respectively [16]. Leukaemic transformation rates at 20 years are estimated at <10% in PV [17].

In contrast, the risk of thrombosis in PV is high: the prevalence of major thrombotic events (arterial events: acute myocardial infarction [AMI], ischaemic stroke, transient ischaemic attack [TIA]; venous events: deep vein thrombosis [DVT], pulmonary embolism [PE], splanchnic thrombosis) at the time of diagnosis ranges approximately 34% to 39%;

corresponding values for thrombosis at follow-up are approximately 8% to 19%. Concerning major thrombotic events, arterial complications occur more often than venous ones. Clinical manifestation of these thrombotic events is mainly discussed only in isolated case reports or case series. A large proportion of patients suffer from vasomotor disturbances (e.g. headache, dizziness, erythromelalgia, acral paraesthesias, atypical chest pain) or pruritus [18]. Some patients may also develop acquired von Willebrand syndrome, especially those with extreme thrombocytosis (PLT >1,000×10^9/L) in both PV and ET and are at risk for acetylsalicylic acid (ASA)-associated bleeding [17]. Thrombosis is a leading cause of morbidity and mortality in PV. The pathogenesis of acquired thrombophilic state in PV is multifactorial and complex. Currently, two main mechanisms are considered as of crucial role: on the one hand, the abnormalities of blood cells (platelets, RBCs, and white blood cells [WBCs]) arising from the clonal proliferation of haematopoietic progenitor cells and the acquisition of a prothrombotic phenotype and on the other hand, the host inflammatory response to cytokines and other mediators secreted by the malignant cells as well as the procoagulant activity of vascular cells triggered by these proinflammatory stimuli [19]. Abnormalities of the clonal proliferation of HSCs include not only quantitative changes but also qualitative modifications that characterize the switch of these cells from a resting to a procoagulant phenotype [20].

3.1.2 STUDY POPULATION AND DATA COLLECTION

To establish our PV database, we relied on two sources. First, we screened medical records (MedSolution) of patients presented at the Haematology Outpatient Unit, 2nd Department of Internal Medicine and Cardiology Centre with the orienting diagnosis of different chronic MPDs, per appropriate ICD (International Classification of Diseases, ICD-10) codes and in a given time period; and second, we checked data of patients subject to molecular diagnostic testing for myeloproliferative disorders at the Department of Medical Genetics, Faculty of Medicine, University of Szeged. Finally, we set up an integrated database including the relevant elements of both data sources.

For our research, medical records of a total of 933 patients were screened. Out of them, the definite diagnosis of PV fulfilling WHO criteria could be established in 108 cases. Data of these 108 subjects made up our “PV Database 1998–2014” (Table 4).

Table 4. *Main demographic and clinicohaematological characteristics of the polycythaemia vera population*

Main characteristics of the PV cohorts	Database 1998–2014
Total number of patients	**108**
Males [N, (%)]	57 (52.8%)
Females [N, (%)]	51 (47.2%)
Age at diagnosis, median (years) (range)	62.6 (24.8–82.0)
Median follow-up (months) (range)	54 (0.0-16.1)
Median Hb (g/L)	174.1 ± 24.0
Median leukocyte count at diagnosis (range) (G/L)	11.2 ± 4.6 (5.2-31.8)
Median platelet count at diagnosis (range) (G/L)	398.8 ± 232.7 (65-1329)
JAK2 V617F-positive cases [N, (%)]	102 (94.4%)
Conventional risk factors in PV	
Age >60 years [N, (%)]	62 (57.4%)
Prior thrombotic events	33 (30.5%)
Low risk [N, (%)]	36 (33.3%)
High risk [N, (%)]	72 (66.7%)

Hb, haemoglobin; JAK2, Janus kinase 2; PV, polycythaemia vera.

Thrombotic events prior to and following the clinical diagnosis were retrospectively collected for each PV patient, with special focus on CV (AMI), cerebrovascular (stroke, TIA, vertebrobasilar insufficiency [VBI]), and venous thrombotic events (DVT, PE, splanchnic vein thrombosis).

Data on CV risk factors present at time of PV clinical diagnosis including hypertension, tobacco use, diabetes mellitus (DM), hyperlipidaemia (hypercholesterolaemia or hypertriglyceridaemia or both), and obesity (body mass index >30 kg/m^2) were also collected (Table 5).

Table 5. *Distribution of cardiovascular risk factors at time of the clinical diagnosis of polycythaemia vera*

Distribution of CV risk factors in PV patients	[N (%)]
Hypertension	73 (67.6%)
Hyperlipidaemia	32 (29.6%)
Diabetes mellitus	23 (21.3%)
Tobacco use	21 (19.4%)
Obesity (BMI >30 kg/m^2)	30 (27.8%)

BMI, body mass index; CV, cardiovascular; PV, polycythaemia vera.

In the haematological management of PV patients, a risk-oriented strategy was adopted: selected low-risk patients received anti-platelet therapy while cytoreductive drugs (e.g. hydroxyurea) in combination with anti-platelet medication were administered to high-risk patients. Phlebotomy was reserved for low-risk patients and for those at high risk before

cytoreductive treatment in order to reach the recommended target haematocrit (Hct) value of <0.45 (Table 6) [21].

Table 6. *Haematological treatment of polycythaemia vera patients*

Haematological treatment of PV patients	[N (%)]
Phlebotomy	51 (47.2%)
Platelet aggregation inhibitor (ASA)	79 (73.1%)
ASA + phlebotomy	41 (38.0%)
Cytoreductive treatment (hydroxyurea)	50 (46.3%)

ASA, acetylsalicylic acid; PV, polycythaemia vera.

3.1.3 STATISTICAL ANALYSES

Clinical data were collected using Microsoft® Excel® 2010 software and subjected to statistical analysis with STATISTICA v9.1 (StatSoft, Hungary) and SPSS 20 (IBM, USA) softwares.

Investigation of the contribution of cardiovascular risk factors in polycythaemia vera

To evaluate and compare the overall effect of CV risk factors present at time of haematological diagnosis, Mann–Whitney *U* tests were performed both for the presence and the absence of thrombotic complications, i.e., CV (AMI), cerebrovascular (ischaemic stroke, TIA, VBI), and venous thrombotic events (DVT, PE, splanchnic vein thrombosis). In addition to each predefined CV risk factor (hypertension, hyperlipidaemia, tobacco use, DM, and obesity), the effect of only one CV risk factor, the effect of two or more CV risk factors as well as the role of leukocytosis (>11.1 G/L) or increased Hct (>45%) were also investigated. Statistical significance was set at 5% and, as reasoned by study population size, also considered at 10%.

To evaluate and compare the probability of thrombosis-free survival for PV patients a) without CV risk factors and with at least one CV risk factor and b) with at most one CV risk factor and with two or more CV risk factors, the Kaplan–Meier method was used, combined with log-rank (Mantel–Cox) tests [22].

3.1.4 LABORATORY METHODS

Samples for *JAK2 V617F* molecular analyses were obtained from the DNA bank of the Department of Medical Genetics based on the information on PV patients diagnosed at the 2nd Department of Internal Medicine and Cardiology Centre between 1998 and 2014. DNA was isolated from ethylenediaminetetraacetic acid (EDTA) -stabilized peripheral blood samples and screened for *JAK2 V617F* mutation using allele-specific polymerase chain reaction (PCR) method as part of the routine diagnostic protocol [23]. For patients whose

haematological diagnosis was established before *JAK2 V617F* mutation screening had become an obligatory part of the diagnostic protocol, samples for genetic testing were collected and analyzed retrospectively.

Serum erythropoietin levels were determined by chemiluminescent immunoassay (Siemens Immulite) at the Laboratory of Endocrinology, University of Szeged [24].

3.1.5 RESULTS

THROMBOTIC EVENTS PRIOR TO AND DURING FOLLOW-UP AND THE MAIN CLINICAL CHARACTERTERISTICS OF POLYCYTHAEMIA VERA PATIENTS WITH OR WITHOUT THROMBOTIC COMPLICATIONS IN THE FOLLOW-UP PERIOD *(Aim I.a)*

The retrospective analysis of all recorded events revealed altogether 33 pre-diagnosis vascular events in 108 (30.5%) patients: 17 cerebrovascular events (stroke/TIA), 8 CV events (AMI), and 8 venous thrombotic events. During the haematological follow-up after the diagnosis of PV, a total of 20 events were observed in 108 (18,5%) patients: 11 cerebrovascular events (stroke/TIA), 7 CV events (AMI), and 2 venous thrombotic events (Table 7).

Table 7. *Vascular events before the clinical diagnosis of polycythaemia vera and during follow-up*

Type of the vascular event	Number of the vascular events
Prediagnostic major vascular events	**33**
Cerebrovascular events (stroke/TIA/VBI)	17
CV events (AMI)	8
Venous thrombotic events	8
Follow-up	**20**
Cerebrovascular events (stroke/TIA/VBI)	11
CV events (AMI)	7
Venous thrombotic events	2

TIA, transient ischaemic attack; VBI, vertebrobasilar insufficiency; CV, cardiovascular; AMI, acute myocardial infarction.

We investigated whether subjects with or without a post-diagnostic history of thrombotic events significantly differ in their main clinical characteristics (Table 8).

Mann–Whitney tests were performed in the cases of the presence or absence of thrombotic events after the diagnosis of PV, comparing the overall effects of series variables: mean follow-up, mean age at diagnosis, *JAK2 V617F*-positivity, haematology blood test results, organomegaly, number of thrombotic events before heamatological diagnosis, vascular risk factors, and treatment. Fisher's exact test results were considered for sex and conventional risk groups. Some CV risk factors as hypertension ($p=0.001$) and tobacco use ($p=0.023$) were significantly different in our two patient groups; the different thrombotic risk of conventional low- and high-risk groups ($p=0.029$) was confirmed as well.

Table 8. *Characteristics of polycythaemia vera patients with or without thrombotic complications during follow-up*

MAIN CLINICAL CHARACTERISTICS	PATIENTS WITH THROMBOTIC COMPLICATIONS	PATIENTS WITHOUT THROMBOTIC COMPLICATIONS	P-VALUE
Number of patients N=108 (100%)	**18**	**90**	
Male [N (%)]	12 (66.7%)	45 (50.0%)	0.198
Female [N (%)]	6 (33.3%)	45 (50.0%)	
Mean follow-up (years)	**3.9 (0.1–11.1)**	**5.8 (0.0–23.0)**	**0.273**
Mean age at diagnosis (years)	**64.9 (38.2–78.5)**	**59.8 (24.8–82.0)**	**0.106**
***JAK2 V617F*-positivity [N (%)]**	**16 (88.9%)**	**86 (95.6%)**	**0.256**
Haematology blood test results at time of haematological diagnosis			
Mean platelet count (G/L)	467.7 ± 274.8	385.0 ± 222.5	0.255
Mean white blood cell count (G/L)	12.7 ± 6.1	10.9 ± 4.3	0.463
Mean red blood cell count (T/L)	6.4 ± 0.8	6.1 ± 1.2	0.059
Mean haemoglobin (g/L)	178.7 ± 27.4	173.1 ± 23.3	0.316
Organomegaly at time of haematological diagnosis			
Hepatomegaly	6 (33.3%)	33 (36.7%)	0.789
Splenomegaly	6 (33.3%)	26 (28.9%)	0.707
Hepatosplenomegaly	2 (11.1%)	19 (21.1%)	0.330
Number of thrombotic events			
Before heamatological diagnosis	6	27	0.937
After heamatological diagnosis	20	0	–
Vascular risk factors			
Hypertension	18 (100.0%)	55 (61.1%)	0.001
Tobacco use	0 (0.0%)	21 (23.3%)	0.023
Diabetes mellitus	5 (27.8%)	18 (20.0%)	0.464
Obesity	8 (44.4%)	22 (24.4%)	0.085
Hyperlipidaemia	8 (44.4%)	24 (26.7%)	0.133
Patient distribution by conventional risk categories			
Low-risk group	2 (11.1%)	34 (37.8%)	0.029
High-risk group	16 (88.9%)	56 (62.2%)	
Treatment			
Hydroxyurea	10 (55.6%)	40 (44.4%)	0.390
Acetylsalicylic acid	14 (77.8%)	65 (72.2%)	0.629
Phlebotomy	10 (55.6%)	41 (45.6%)	0.440

CARDIOVASCULAR AND CEREBROVASCULAR COMPLICATIONS *(Aim I.b)*

Cardiovascular complications

Detailed clinical data and coronary angiography findings for adequate cardiological analyses of CV complications in PV patients during follow-up were available for 6 patients (1 male, 5 females; mean age 69.5 years [range: 64–76 years]).

Mean Hct was 52.8 ± 8.6% at time of haematological diagnosis and 49.5 ± 7.9% at the onset of AMI. Median WBC count was 11.28 ± 5.7 G/L at haematological diagnosis while by the onset of AMI, it increased to a level of 13.47 ± 5.8 G/L.

Five (83.3%) out of the six analyzed patients exhibited *JAK2 V617F* mutation. Most of the patients (83.3%) had at least two major conventional CV risk factors.

Non–ST-segment elevation myocardial infarction (NSTEMI) was diagnosed in all six PV patients by coronary angiography. Significant stenosis of coronary arteries requiring percutaneous coronary intervention with stent implantation was seen in two patients. Coronary angiography showed only normal epicardial coronary arteries, non-significant stenosis, or distal occlusion in one patient each. One patient underwent coronary artery bypass grafting and saphenous vein graft stenting. Evaluated cases are listed individually in Table 9.

Table 9. *Presentation of individual polycythaemia vera patients with cardiovascular complications enrolled in the detailed analyses*

CASE NO. AGE/SEX/DATE OF DIAGNOSIS	TIME BETWEEN CARDIOLOGICAL EVENT AND PV DIAGNOSIS	CV RISK FACTORS PRESENT AT PV DIAGNOSIS	*JAK2 V617F* MUTATION	CARDIOLOGICAL COMPLICATIONS		HAEMATOLOGICAL TREATMENT AFTER PV DIAGNOSIS
				CARDIOLOGICAL PRESENTATION	CORONARY ANGIOGRAPHY FINDINGS	
CASE 1 72/M/2005	8 months	hypertension, hyperlipidaemia, obesity	negative	NSTEMI	LAD: diagonal borderline lesion LCX: first OM branch 20% stenosis RC: ostial 80% stenosis (PCI + stent implantation)	ASA + clopidogrel + phlebotomy
CASE 2 63/F/2010	15 months	hypertension	*positive*	NSTEMI	LAD: proximal 90% stenosis (PCI + stent implantation) LCX: normal RC: 50% stent stenosis	ASA + clopidogrel
CASE 3 74/F/2005	41 months	hypertension, hyperlipidaemia	*positive*	NSTEMI	LAD: proximal 40% stenosis LCX: normal RC: normal	Hydroxyurea + phlebotomy
CASE 4 76/F/2009	13 months	hypertension, hyperlipidaemia, obesity, DM	*positive*	NSTEMI	LAD: ostial occlusion, LIMA-LAD (normal) LCX: 95% stenosis, proximal 70% stenosis of SVG-LCX (stent in SVG) RC: proximal occlusion (CABG)	ASA + phlebotomy
CASE 5 64/F/2013	8 months	hypertension, obesity	*positive*	NSTEMI	LAD: normal LCX: normal RC: normal	ASA
CASE 6 68/F/2011	4 months	hypertension, obesity, DM	*positive*	NSTEMI	LAD: normal LCX: normal RC: distal occlusion	ASA + hydroxyurea

ASA, acetylsalicylic acid; CABG, coronary artery bypass grafting; CV, cardiovascular; DM, diabetes mellitus; F, female; LAD, left anterior descending coronary artery; LCX, left circumflex coronary artery; LIMA, left internal mammary artery; LM, left main coronary artery; M, male; NSTEMI, non–ST-segment elevation myocardial infarction; OM, obtuse marginal artery; PCI, percutaneous coronary intervention; PV, polycythaemia vera; RC, right coronary artery; SVG, saphenous vein graft.

Cerebrovascular complications

Detailed analysis of cerebrovascular events was performed in 11 out of the 108 PV patients enrolled (9 males, 2 females; median age: 65 years [range: 52–79 years]). *JAK2 V617F* mutation positivity was detected in 10 of the 11 analyzed cases (91%). Most of the patients (8/11, 72%) had at least two major conventional vascular risk factors (e.g. hypertension, hyperlipidaemia, DM, or obesity).

Mean Hct level at onset of cerebrovascular complications after the initiation of specific haematological treatment was lower (45% [range: 41–57%]) than at PV haematological diagnosis (54% [range: 45–66%]). Mean WBC at time of haematological diagnosis was 13 G/L (range: 5–24 G/L) and persisted (13 G/L [range: 5–25 G/L]) during the course of post-treatment cerebrovascular thrombotic events.

In most of the cases (7/11 patients, 63%), chronic ischaemic white matter lesions were detected on brain computed tomography scan obtained at cerebrovascular event onset. In addition, mild cerebral atrophy was also a frequent finding. The clinical presentation was predominated by lacunar syndromes or VBI. Two patients – one of them on anticoagulant therapy – sustained haemorrhagic stroke. Overall, these data allow us to suppose that after adjusting for major conventional vascular risk factors, PV predisposes to small vessel cerebral disease manifested mainly as lacunar syndromes, even if most of the patients had additional vascular risk factors, too. Evaluated cases are listed individually in Table 10.

Table 10. *Main characteristics of polycythaemia vera patients with cerebrovascular event*

CASE NO. AGE/SEX/DATE OF DIAGNOSIS	CV RISK FACTORS	CEREBROVASCULAR COMPLICATIONS		TREATMENT	
		NEUROLOGICAL PRESENTATION	CT/MRI FINDINGS	AFTER PV DIAGNOSIS	AFTER THE FIRST CEREBROVASCULAR EVENT
CASE 1 **72/M/2005**	hypertension, hyperlipidaemia, obesity	**2011:** VBI	**CT:** mild cerebral atrophy medium-sized chronic ischaemic white matter lesions	ASA + phlebotomy	clopidogrel + phlebotomy
CASE 2 **64/M/2010**	hypertension, hyperlipidaemia, DM	**2011:** VBI	**CT:** no pathological lesion **MRI** (2005): mild chronic ischaemic white matter lesions	ASA + phlebotomy	clopidogrel + hydroxyurea
CASE 3 **70/M/2008**	hypertension, hyperlipidaemia, *MTHFR C677T* homozygous polymorphism (with currently normal homocystein levels)	**05/2011:** left MCA ischaemic stroke (mild), dementia (mixed) **11/2011:** left hemispheric haemorrhagic stroke	**CT** (05/2011): mild cerebral atrophy, lacunes in left basal ganglia, mild chronic left-sided ischaemic white matter lesions **CT** (11/2011): acute parenchymal haemorrhage (3×6 cm) in the left parieto-temporal region (concomitant anticoagulant therapy for AF); left MCA stenosis	ASA + phlebotomy + *(warfarin for AF)*	ASA + hydroxyurea + *anticoagulant treatment (with low-therapeutic INR)*
CASE 4 **79/M/2001**	hypertension	**2008:** vertigo, suspected VBI	**CT:** mild cerebral atrophy, some lacunes in basal ganglia, mild chronic ischaemic white matter lesions	ASA + phlebotomy + hydroxyurea	ASA + phlebotomy + hydroxyurea
CASE 5 **53/M/2003**	hypertension, hyperlipidaemia, obesity	**2007:** VBI **2010:** VBI	**CT:** not available **CT:** mild cerebral atrophy	ASA + phlebotomy	ASA + phlebotomy + hydroxyurea + pentoxifylline
CASE 6 **65/M/2011**	hypertension, DM	**2013:** right MCA stroke (mild)	**CT:** acute infarction (2×2 cm) in right hemispheric white matter	phlebotomy	*(warfarin for AF)*
CASE 7 **76/M/2006**	hypertension, obesity	**2012:** left MCA stroke (mild)	**CT:** medium-degree cerebral atrophy, chronic periventricular white matter lesions	ASA +hydroxyurea + *(acenocoumarol for AF)*	clopidogrel + *(acenocoumarol for AF)*
CASE 8 **57/M/2008**	hypertension, hyperlipidaemia, DM, obesity	**2008:** right MCA stroke (mild) **2010:** worsening of chronic neurological signs (dysarthria)	**CT** (2008, 2010): cerebral atrophy, lacunes in basal ganglia, extensive chronic ischaemic white matter lesions	ASA	clopidogrel
CASE 9 **52/F/1998**	hypertension, PAD	**2004:** TIA (VBI)	**CT:** negative	ASA + hydroxyurea *(acenocoumarol)*	hydroxyurea + clopidogrel + *(acenocoumarol)*
CASE 10 **77/M/2012**	hypertension	**2012:** fatal right MCA haemorrhagic stroke	**CT:** right-sided space-occupying haemorrhage in basal ganglia with intraventricular extension, chronic white matter lesions	ASA	
CASE 11 **54/F/2013**	hypertension, hyperlipidaemia, obesity	**2014:** vertigo – VBI	**CT:** not available	ASA	clopidogrel

AF, atrial fibrillation; ASA, acetylsalicylic acid; CT, computed tomography; CV, cardiovascular; DM, diabetes mellitus; F, female; M, male; MCA, middle cerebral artery; MRI, magnetic resonance imaging; PAD, peripheral arterial disease; PCA, posterior cerebral artery; PV, polycythaemia vera; TIA, transient ischaemic attack; VBI, vertebrobasilar insufficiency.

THE CONTRIBUTION OF CARDIOVASCULAR RISK FACTORS TO SUBSEQUENT THROMBOTIC COMPLICATIONS *(Aim I.c)*

Univariate analyses revealed a significant overall association between thrombotic complications and high blood pressure (p=0.000), tobacco use (p=0.014), and obesity (p=0.078). Hyperlipidaemia (p=0.112) and DM (p=0.323) were not associated with an increased risk of subsequent thrombosis. The presence of one CV risk factor (p=0.016) or two or more CV risk factors (p=0.024) significantly increased the occurrence of thrombotic complications. Leukocytosis (WBC >11.1 G/L), however, did not increase significantly the risk of thrombotic events (p=0.119). The frequency of thrombotic events during follow-up differed significantly between PV subgroups with Hct values below or above 45% (p=0.089) (Table 11).

Table 11. *Mann–Whitney U test results in subgroups of polycythaemia vera patients sustaining or not sustaining thrombotic events during follow-up*

Comparison of PV patients who did or did not sustain thrombotic events during follow-up	
VARIABLES	**MANN–WHITNEY UNIVARIATE ANALYSIS p-VALUE**
CV risk factors	
Hypertension	0.000**
Hyperlipidaemia	0.112
Tobacco use	0.014**
Diabetes mellitus	0.323
Obesity	0.078*
Presence of 0 or 1 CV risk factor	0.016**
Presence of ≥2 CV risk factors	0.024**
White blood cell count >*11.1 G/L*	0.119
Haematocrit >45%	0.089*

*Significant differences at 10% are marked by * and those at 5% by **.*
CV, cardiovascular; PV, polycythaemia vera.

To compare the thrombosis-free survival of patients in the presence or absence of the investigated CV risk factors, Kaplan–Meier curves and log-rank tests (Mantel–Cox) were used which indicated a significant difference of the thrombosis-free survival between PV patients without CV risk factors (N=20) and those with at least one CV risk factor (N=88) (p=0.017) (Figure 1).

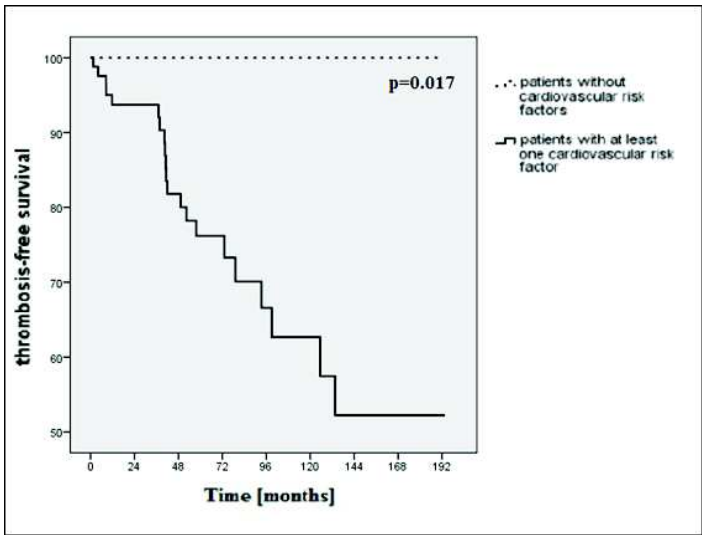

Figure 1. *Probability of thrombosis-free survival during haematological follow-up in subgroups of polycythaemia vera patients without cardiovascular risk factors and with at least one cardiovascular risk factor*

A significant difference was also observed between PV patients with at most one CV risk factor (N=49) and PV patients with two or more CV risk factors (N=59) (p=0.011) (Figure 2).

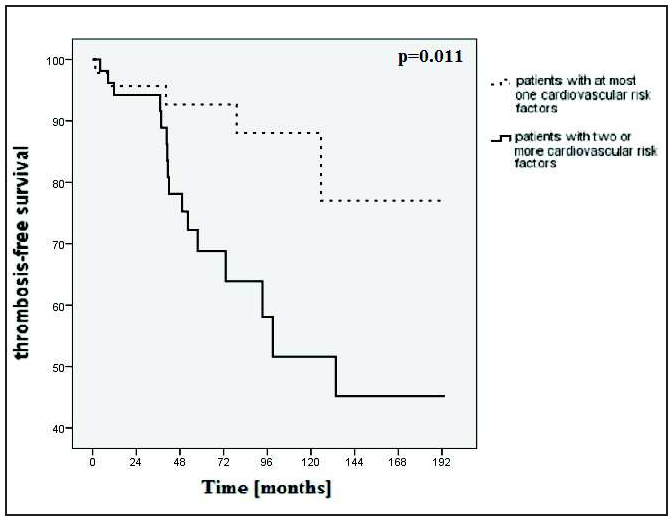

Figure 2. *Probability of thrombosis-free survival during haematological follow-up in subgroups of polycythaemia vera patients with at most one cardiovascular risk factor and with two or more cardiovascular risk factors*

In another complex overview, similar results were obtained for CV risk factors in female patients with MPDs; however, the study population for these analyses was defined as the sum of female patients with ET or PV [25].

3.2 SYSTEMIC MASTOCYTOSIS

3.2.1 GENERAL CONSIDERATIONS AND BACKGROUND OF THE SYSTEMIC MASTOCYTOSIS STUDY

Mastocytosis represents one of the eight subcategories of MPNs in the 2008 WHO classification of myeloid neoplasms and acute leukaemia. It is an orphan disease characterized by the pathological accumulation of morphologically and immunophenotypically abnormal mast cells in one, two, or more organ systems. Organ systems most often involved are the bone marrow, skin, liver, and gastrointestinal tract [26]. The clinical presentation of mastocytosis is heterogeneous, ranging from skin-limited disease (cutaneous mastocytosis [CM]) affecting particularly children that may spontaneously regress to varying degrees of extracutaneous involvement (SM) generally seen in adults that may be associated with multiorgan dysfunction and a reduced survival [27-30].

The clinical course of SM varies from an asymptomatic form (indolent SM [ISM]) to a highly progressive type (aggressive SM [ASM]) or even mast cell leukaemia (MCL) [31].

The advanced features of the 2008 WHO classification are reflected not only in its novel, molecular-based nomenclature and clear diagnostic criteria supporting the differentiation between each subcategory but also in its high prognostic relevance for SM. In the ever largest clinical trial to validate this correlation, Lim *et al.* found that, compared to subjects with ASM or SM with an associated clonal haematological non-mast cell lineage disease (SM-AHNMD), ISM patients had a significantly better prognosis in terms of overall survival and leukaemia-free survival. Furthermore, there was no significant difference between the life expectancy of ISM patients and the age- and sex-matched American (USA) population for the appropriate time period, based on the date of diagnosis [27].

According to their clinicopathological features, the revised 2008 WHO classification distinguishes several subcategories within the group of MCDs.

As per the 2008 WHO criteria outlined in Tables 12–13, the following categories of SM are defined: ISM, SM-AHNMD, ASM, and MCL. The diagnosis of SM can only be confirmed after the identification of morphological, immunophenotypic, and/or mutational characteristics of the neoplastic mast cells in an extracutaneous tissue, usually in the bone marrow. In addition, the WHO classification includes CM and rare, localized mast cell tumours, namely mast cell sarcoma (MCS) and extracutaneous mastocytoma. These entities do not fall under the category of SM and were therefore not included in our research aimed exclusively at the investigation of SM cases.

Table 12. *WHO classification of mastocytosis [13, 30, 32]*

1. Cutaneous mastocytosis (CM) a. Urticaria pigmentosa/maculopapular CM (UP/MPCM) b. Diffuse CM (DCM) c. Solitary mastocytoma of the skin
2. Indolent systemic mastocytosis (ISM): Meets criteria for SM. No C findings. No evidence of associated clonal haematological non-mast cell lineage disease. a. Smouldering SM (SSM): As above (ISM) but with two or more B findings and no C findings. b. Isolated bone marrow mastocytosis: As above (ISM) with bone marrow involvement but without skin involvement.
3. SM with an associated clonal haematological non-mast cell lineage disease (SM-AHNMD): Meets criteria for SM and criteria for AHNMD as a distinct entity per WHO classification.
4. Aggressive SM (ASM): Meets criteria for SM. One or more C findings. No evidence of MCL.
5. Mast cell leukaemia (MCL): Meets criteria for SM. Bone marrow biopsy shows a diffuse infiltration, usually compact, by atypical immature mast cells. Bone marrow aspirate smears show ≥20% mast cells. In typical MCL, mast cells account for ≥10% of peripheral blood white cells. Rare variant: aleukaemic MCL.
6. Mast cell sarcoma (MCS)
7. Extracutaneous mastocytoma

CM, cutaneous mastocytosis; ASM, aggressive systemic mastocytosis; MCL, mast cell leukaemia; MCS, mast cell sarcoma; UP, urticaria pigmentosa; AHNMD, associated clonal haematological non-mast cell lineage disease.

Table 13. *B and C findings in advanced mastocytosis*

B findings 1. Bone marrow biopsy showing >30% infiltration by mast cells (focal, dense aggregates) and/or serum total tryptase level >200 ng/mL. 2. Signs of dysplasia or myeloproliferation in non-mast cell lineage(s), but insufficient criteria for definitive diagnosis of a haematopoietic neoplasm (AHNMD), with normal or slightly abnormal blood counts. 3. Organomegaly: palpable hepatomegaly, splenomegaly, or lymphadenopathy (on CT or US >2 cm) without impaired organ function.
C findings 1. Cytopenia(s) (ANC $<1.0\times10^9$/L, Hb <10 g/dL, or PLT $<100\times10^9$/L) but no obvious non-mast cell haematopoietic malignancy. 2. Palpable hepatomegaly with impairment of liver function, ascites, and/or portal hypertension. 3. Skeletal involvement with large osteolytic lesions and/or pathological fractures. 4. Palpable splenomegaly with hypersplenism. 5. Malabsorption with weight loss due to gastrointestinal mast cell infiltrates.

AHNMD, associated clonal haematological non-mast cell lineage disease; ANC, absolute neutrophil count; CT: computed tomography; Hb, haemoglobin; PLT, platelet; US, ultrasonography.

The diagnosis of SM can be made when the major criterion and ≥1 minor criterion, OR ≥3 minor criteria are present (Table 14).

Table 14. *Schematic overview of the 2008 WHO diagnostic criteria of systemic mastocytosis[30] [33]*

Major criterion
Multifocal, dense infiltrates of mast cells (≥15 mast cells in aggregates) detected in bone marrow and/or other extracutaneous organs
Minor criteria
a. >25% of the mast cells in bone marrow or other extracutaneous organ(s) show an abnormal morphology in bone marrow smears or in histologies
b. *KIT* mutation at codon 816 in extracutaneous organ(s)
c. Mast cells in bone marrow express CD2 and/or CD25
d. Serum total tryptase >20 ng/ml (does not count in patients with AHNMD-type disease)

AHNMD, associated clonal haematological non-mast cell lineage disease.

Although there is no internationally accepted, universal definition for orphan diseases, the different criteria are common in their use of prevalence rates. The prevalence threshold varies widely between the European Union, the USA, and Japan. According to the definition of the European Committee for Orphan Medicinal Products, severe or life-threatening conditions with a prevalence of less than 5:10,000 are considered as orphan diseases [34, 35].
Up to now, there are only very limited data on the incidence and prevalence of mastocytosis. Therefore, ECNM has established a registry to collect information from a number of patients suffering from this rare disease [36]. Within this program, an incidence of 5 to 10 cases per 1,000,000 person-years was obtained [33].
The prevalence of mastocytosis in Central Europe is estimated at 0.5-1.0:10,000 [37]. Population-based epidemiological data and local/regional data on ISM have been reported from Denmark and The Netherlands (Groningen) but ECNM is still collecting data from ten European countries [36, 38, 39]. ISM is the most frequent subtype of SM in adults. It is predominated by cutaneous manifestations (UP) but recurrent systemic symptoms (e.g. flushing, palpitations, muscle cramps, abdominal pain, diarrhoea, bone pain) related to mast cell degranulation and mediator release and/or allergies or anaphylaxis may also occur. Factors which may lead to mast cell activation include heat, cold, stress (physical or emotional), medications, insect bites, and food or are idiopathic. The symptoms may have a strong negative impact on the quality of life while anaphylactic reactions can be severe or even fatal [26, 38].

On the contrary, symptoms in ASM (e.g. cytopenia, ascites, malabsorption, or osteolytic skeletal lesions) arise from organ dysfunction due to mast cell infiltration. SM-AHNMD is characterized by the presence of another clonal haematological disease such as myelodysplastic syndrome (MDS), myeloid leukaemia or another MPN, or non-Hodgkin lymphoma concomitant to SM [26, 38]. Mast cells are tissue resident cells of HSC origin. The differentiation and survival of mast cells is mainly regulated by the activation of KIT by its ligand stem cell factor [40, 41] Most of the adult patients suffering from mastocytosis, regardless of disease subtype, harbour the somatic activating mutation of the oncogenic receptor tyrosine kinase *KIT* gene (exon 17, D816V) [33, 42-45] The *KIT D816V* mutation, which is found in up to 85% of all SM patients, is of great pathogenetic and diagnostic relevance [26, 43-45]

3.2.2 *PATIENTS AND DATA COLLECTION*

Between 2001 and 2013, a total of 35 patients were diagnosed with SM (20 males, 15 females; median age: 57 years [range: 31–85 years]) in our centre: 14 with ISM, 15 with SM-AHNMD, and 6 with ASM subtypes, respectively (Table 15). In the investigated period, no other MCD entities like MCL, extracutaneous mastocytoma, or MCS occurred. Out of all registered cases, only those who strictly fulfilled the 2008 WHO criteria for SM (major criterion and ≥1 minor criterion, OR ≥3 minor criteria) were considered to be enrolled in our research.

In Hungary, serum tryptase determination is currently not available; however, all our presented cases met the 2008 WHO criteria, even without known tryptase levels. The haematological management of each particular patient used to be based on the current treatment standards available at time of diagnosis and therapy initiation [32, 46-53].

Table 15. *Summary of the main demographic and disease-related characteristics of the investigated sytemic mastocytosis study population by disease subtype*

Characteristics	ISM	SM-AHNMD	ASM
Main demographic characteristics			
Patients (N)	14	15	6
Males (N)	8	7	4
Females (N)	6	8	2
Median age at diagnosis (years) (range)	55 (31–81)	57 (34–72)	65 (54–85)
Median follow-up (months) (range)	50.5 (5–240)	25 (1–104)	20.5 (2–35)
Disease-related characteristics			
Associated haematological disease (N)	–	MDS (3) AML (3) MF (2) ET (1) PV (1) CML (1) iHES (1) MM (1) PTCL (1) B-ALL (1)	–
Urticaria pigmentosa (N)	8/14	–	–
Mediator-related symptoms (N)			
skin (flush, pruritus)	5/14	–	2/6
gastrointestinal (diarrhoea)	3/14	–	–
cardiovascular (palpitation, dizziness, syncope)	2/14	–	–
neurological	1/14	–	1/6
anaphylaxis	2/14	–	–
Coexistent allergy (N) (inhalation, nutritive, drug, insect venom)	3/14	–	–
Constitutional symptoms (N) (generalized weakness, fatigue, sweats, chills, arthralgia, myalgia)	1/14	1/15	6/6
Organ damage/Organopathy (N)			
Hepatomegaly /	2/14	3/15	
*hepatomegaly with elevated alkaline phosphatase	1/14*		6/6*
Splenomegaly	–	2/15	3/6
Adenopathy	1/14	1/15	3/6
Osteopenia/osteoporosis/osteolysis	3/14	–	3/6

AML, acute myeloid leukaemia; ASM, aggressive systemic mastocytosis; B-ALL, B-cell acute lymphoblastic leukaemia; CML, chronic myeloid leukaemia; ET, essential thrombocythaemia; iHES, idiopathic hypereosinophilic syndrome; ISM, indolent systemic mastocytosis; MDS, myelodysplastic syndrome; MF, myelofibrosis; MM, multiple myeloma; PTCL, peripheral T-cell lymphoma; PV, polycythaemia vera; SM-AHNMD, systemic mastocytosis with an associated clonal haematological non-mast cell lineage disease.

3.2.3 STATISTICAL ANALYSES

Evaluation of the survival probability in systemic mastocytosis

The survival probability in the various SM subgroups (ISM, AHNMD, ASM) was estimated by Kaplan–Meier analyses. Calculated patient survival rates were compared to the expected survival data of age- and sex-matched Hungarian population controls obtained from the Hungarian Central Statistical Office. Age- and sex-matched survival statistics were retrieved from life tables based on a population of 1,000 newborns [22]. For each year, the incidence rate was calculated as the number of new cases divided by the mid-year population size. The latter was obtained as the mean of the population sizes on 1st January of the relevant year and the next year. The cumulative incidence for 13 years was computed as $CI = 1 - e^{-\sum_{i=1}^{13} IR_i \cdot t_i}$, where IR_i denotes the yearly incidence rates from the first to the thirteenth year, and t_i denotes the length of each time period which is one year in this case for all the 13 periods.

3.2.4 LABORATORY METHODS

Our haematological centre – operating as an integrated part of the 2nd Department of Internal Medicine and Cardiology Centre – is a regional haematological diagnostic and treatment centre catering for a population of approx. 1,103,463 inhabitants in south-eastern Hungary (Figure 3). Our data were in part retrieved from the outpatient and inpatient database of our centre sorted by ICD code and in part obtained from bone marrow biopsy reports released by the Laboratory of Tumour Pathology and Molecular Diagnostics, Szeged. All paediatric patients with CM and adult patients presenting with skin lesions who refused bone marrow biopsy were not included in our study.

Figure 3. *Geographical location of our regional centre catering for the population of three south-eastern counties of Hungary (South Great Plain region).*

Source: http://www.ksh.hu/regional_atlas_counties?lang=en

All bone marrow test results released since 2001 were screened for SM and re-assessed by morphological, immunohistochemical and molecular (PCR) methods, according to the 2008 WHO criteria.

We considered only those cases as SM which strictly fulfilled the relevant 2008 WHO criteria (major criterion and ≥1 minor criterion, OR ≥3 minor criteria). In Hungary, serum tryptase determination is currently not available; however, all our presented cases met the 2008 WHO criteria, even without known tryptase levels. C-*KIT* mutation analysis is routinely performed on every bone marrow biopsy sample evaluated at our centre. *C-KIT* mutations detected by PCR method in this patient population were confirmed by Sanger sequencing. Out of all detected cases, only those were incuded in our study database which strictly complied with the relevant 2008 WHO criteria (major criterion and ≥1 minor criterion, OR ≥3 minor criteria).

Immunohistochemistry

Bone marrow trephine biopsies were fixed in neutral buffered formaldehyde supplemented with methanol and glucose (Schaffer's fixative), decalcified in 12.5% (w/v) pH 7.0 EDTA solution (Sigma-Aldrich), and embedded into paraffin. The immunohistochemical reactions were executed on 2-4 μm thick formaldehyde-fixed and paraffin-embedded (FFPE) sections waxed in xylene and graded ethanol, and pretreated by heat-induced antigen retrieval. The following primary antibodies were used: anti-CD117 (Dako, Denmark A/S), anti-CD25, anti-mast cell tryptase, anti-CD68 (Leica Biosystems/Novocastra), and anti-phospho-STAT5 (Santa Cruz Biotechnology, USA). Detection was carried out with Novolink polymer kit (Leica Biosystems/Novocastra) according to the manufacturer's instructions while nuclear staining was completed with Mayer's haematoxylin.

DNA isolation, PCR amplification, and DNA sequencing

Molecular tests were performed on crude DNA lysates made from FFPE tissue sections. Briefly, ten pieces of paraffin sections of 10 μm thickness per each bone marrow trephine biopsy sample were placed into sterile 1.5 ml Eppendorf tubes, mounted with 100 μl lysis buffer (50 mM Tris-HCl, 1.5 mM $MgCl_2$, pH 8.0) containing 10 μl proteinase K (PK) solution (20 mg/ml, MBI Fermentas Life Sciences), centrifuged at 13,000 rpm for 3 min, and incubated at 56 ºC for 18 h. Afterwards, PK was inactivated at 96 ºC for 15 min and centrifuged at 13,000 rpm for 3 min. The retrieved supernatant was used as template in 1:10–

1:20 dilution. PCR amplification was carried out in 25 µl reaction mixture in an Eppendorf Mastercycler® gradient thermal cycler. PCR parameters were as follows: 100 µM dNTP (MBI Fermentas Life Sciences), 1.75 mM $MgCl_2$, 25 pmol/µl of each primer, 2 µl DNA template, and 1.5 IU recombinant *Taq* polymerase (MBI Fermentas Life Sciences) per reaction. The following primers were used: C-KIT-outer-Fo 5'-GCCAGAAATATCCTCCTTACTCA-3', C-KIT-allele-specific-Fo 5'-GTGATTTTGGTCTAGCCAGCKT-3', C-KIT-Re 5'-GTTGAAACTAAAAATCCTTTGCAGGAC-3'. The temperature and timing parameters of the cycles were as follows: denaturation at 95 ºC for 30 s, annealing at 56 ºC for 30 s, extension at 72 ºC for 30 s, last extension at 72 ºC for 10 min. PCR products were run on 12.5% polyacrylamide gel using the Mini PROTEAN® Tetra Cell (Bio-Rad Laboratories) electrophoresis set and visualized with $AgNO_3$ staining. This primer set generated a 153-basepair-long outer PCR product used as reaction control and a 111-basepair-long mutation-specific product. PCR products of expected size were Sanger sequenced using Applied Biosystems® 3500 DX series genetic analyser and evaluated with the free Sequence Scanner software (v1.0). The sequences obtained were run against the BLAST database.

In one single case [54], we screened our patient by Sanger sequencing for the most frequently reported *KIT* mutations in exons 9, 11, and 17 in a close co-operation with the 1st Department of Pathology and Experimental Cancer Research, Semmelweis University, Budapest [54].

3.2.5 *RESULTS*

CLINICOPATHOLOGICAL FINDINGS ALONG WITH BONE MARROW HISTOLOGICAL FEATURES, MOLECULAR CHARACTERISTICS, AND LABORATORY PARAMETERS AT PRESENTATION IN A LARGE COHORT OF SYSTEMIC MASTOCYTOSIS PATIENTS (*Aim II.a)*

In the ISM group, bone marrow biopsy analysis revealed ISM in 14 patients (8 males and 6 females) with a median age of 55 years (range: 31–81 years). The median duration of follow-up was 50.5 months (range: 5–240 months). Cutaneous manifestation (UP) was detected in 57% (8/14) of the patients while mediator-related symptoms occurred in 28% (4/14) of them. 78% (11/14) of the ISM patients were positive for *KIT D816V* mutation.

A total of 15 patients (7 males and 8 females; median age: 57 years [range: 34–72 years]) were diagnosed with AHNMD. The median follow-up time in this subgroup was 25 months (range: 1–104 months). Bone marrow biopsy was done as required by signs of the associated neoplasm such as bone lesions or clinically significant peripheral blood count abnormalities, e.g. eosinophilia or elevated or decreased WBC or PLT counts. This subtype of

SM was associated with MDS or acute myeloid leukaemiain three cases each, with MF in two cases, or with ET, PV, CML, HES, multiple myeloma, peripheral T-cell lymphoma, or B-cell acute lymphoblastic leukaemia in one case each. In these patients, no cutaneous lesions or mediator-related symptoms were observed. *KIT D816V* mutation positivity was detected in 80% (12/15) of the patients.

ASM was diagnosed in 6 patients (4 males and 2 females) with a median age of 65 years (range: 54–85 years). The median follow-up was 20.5 months (range: 2–35 months). Bone marrow biopsy was performed for hepatosplenomegaly with or without pancytopenia/anaemia/eosinophilia and weight loss. All ASM patients presented with at least one C finding, as defined by the 2008 WHO criteria (marked cytopenia, osteolysis with or without pathological fractures, ascites and elevated liver enzymes, malabsorption with hypoalbuminaemia, palpable splenomegaly with hypersplenism). Cutaneous lesions were detected in 33% (2/6) of the patients. Mediator-related symptoms occurred in one case. *KIT D816V* mutation positivity was confirmed in 83% (5/6) of the patients.

Clinical symptoms, immunophenotypes as well as PCR and Sanger sequencing results for each patient are summarized in *Supplement 2* to this dissertation (*Appendix III*) and the subgroup distribution (ISM; AHNMD, ASM) and detailed characteristics of these patients have been recently published in *Supplement 1 (Appendix III)* to the *Clinical and Molecular Diagnostic Evaluation of Systemic Mastocytosis in the South-Eastern Hungarian Population Between 2001–2013 – A Single-centre Experience* by Imelda Marton *et al.* [55].

FREQUENCY OF *KIT D816V* MUTATION *(Aim II.b)*

KIT D816V mutation positivity was detected in 78% (11/14) of ISM patients, 80% (12/15) of AHNMD patients and 83% (5/6) of ASM patients, respectively. Main patient characteristics including bone marrow biopsy test PCR and Sanger sequencing results results are presented in *Supplement 2* to this dissertation (*Appendix III*) [55].

LIFE EXPECTANCY IN SYSTEMIC MASTOCYTOSIS *(Aim II.c)*

Overall disease-specific survival of SM patients was analyzed by Kaplan–Meier method and is demonstrated in Figure 4.

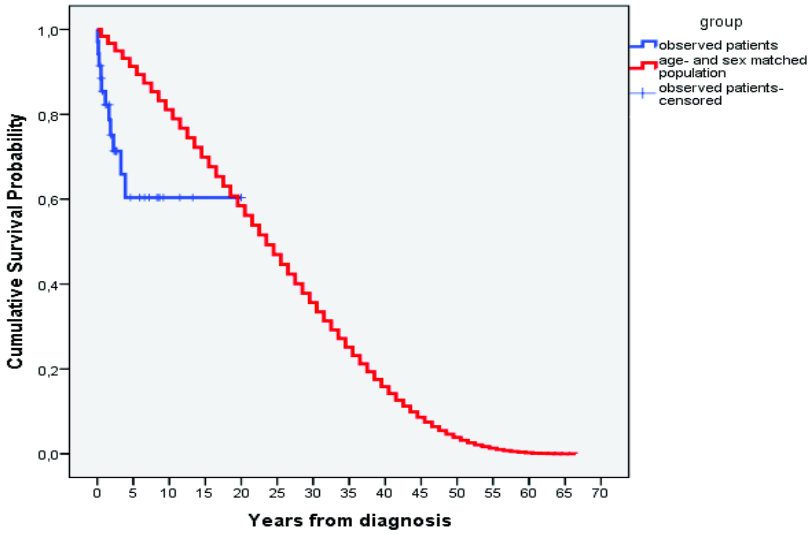

Figure 4. *Kaplan–Meier survival curve demonstrating cumulative survival probability of patients with systemic mastocytosis. The survival observed in SM patients (blue) is compared to the expected survival of the age- and sex-matched Hungarian population (red).*

Similarly, survival data for each SM subtype were also generated by Kaplan–Meier analysis and are presented in Figure 5.

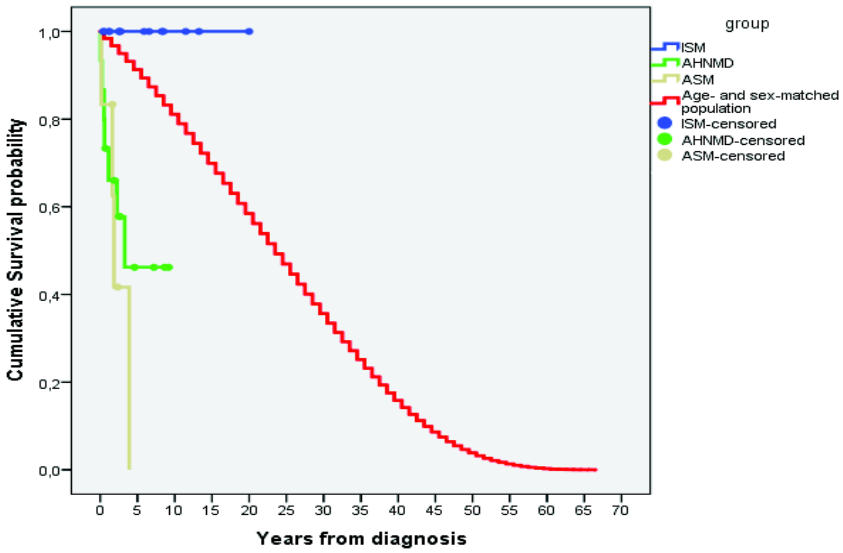

Figure 5. *Survival of systemic mastocytosis patients by disease subtype. Kaplan–Meier survival rates of SM patients classified by disease subtype – ISM (blue), AHNMD (green), and ASM (yellow) – were compared to the expected survival of the age- and sex-matched Hungarian population (red).*

The median survival in the ASM group was 1.73 years while the survival time of the AHNMD patients did not reach a median during follow-up. None of the patients died during the follow-up period in the ISM subpopulation. The median survival for the age- and sex-matched control population was 23.5 years. The comparison of the survival curves using Mantel–Cox, Breslow and Tarone–Ware tests uniformly resulted in a p-value of 0.000 indicating significantly different survival patterns in the evaluated SM subgroups.

Moreover, it is important to emphasize that our research identified an uncommon case in the investigated patient population. In our case report [54], we demonstrated a female ISM patient with recurrent cutaneous symptoms and a follow-up time of 27 years. This case illustrates that in selected cases, imatinib mesylate could be a good choice to achieve a reduction of skin lesions, like in this *KIT D816V*-negative patient. The case is presented in details in *Appendix IV* to this dissertation as the publication of Imelda Marton *et al.*: *Therapeutic challenge during the long-term follow-up of a patient with indolent systemic mastocytosis with extensive cutaneous involvement.*

CUMULATIVE INCIDENCE OF SYSTEMIC MASTOCYTOSIS *(Aim II.d)*

Our regional diagnostic and treatment centre receives SM patients from the population of south-eastern Hungary, representing a total of 1,103,463 inhabitants [56]. These data allowed us to calculate the cumulative incidence of SM for 13 years in the general population, which proved to be 0.27/10,000 in this region.

Four patients (Cases 2, 13, 29, and 35) were excluded from this estimation of the cumulative incidence: although they were diagnosed in our centre, were inhabitants of another administrative region of Hungary.

3.3 HYPEREOSINOPHILIC SYNDROME

3.3.1 GENERAL CONSIDERATIONS AND BACKGROUND OF THE HYPEREOSINOPHILIC SYNDROME STUDY

Traditionally, peripheral blood eosinophilia was classified as mild (absolute eosinophil count [AEC] from upper limit of normal to 1,500/mm^3), moderate (AEC 1,500–5,000/mm^3), and severe (AEC >5,000/mm^3). Hypereosinophilia defined as AEC >1,500/mm^3 may be asssociated with tissue damage [57-60]. The current definitions and criteria of eosinophilic disorders and related syndromes are often overlapping, both with each other and within the area of several disciplines like pathology, haematology, immunology, and allergology. Hence,

the establishment of multidisciplinary definitions along with refined criteria for the various forms of hypereosinophilia has become essential [61]. If any secondary cause of eosinophilia can be excluded, the condition is classified as either clonal or idiopathic primary eosinophilia, depending on the presence or absence of a molecular, cytogenetic, or histological evidence for a myeloid malignancy [59].

The classification of eosinophilic diseases has been revised by the updated 2008 WHO scheme (Table 1). CEL-NOS is one of the eight subcategories within MPNs. Reflecting the growing number of recurrent, molecularly defined primary eosinophilias, a new major category of "myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1*" has been generated [62]. Idiopathic HES represents a distinct entity (Table 16) .

The rearranged *PDGFRA/B* and *FGFR1* fusion genes encode constitutively activated tyrosine kinases. Out of them, *PDGFRA/B*-rearranged neoplasms with eosinophilia are imatinib-sensitive. Therefore, this classification has a direct therapeutical relevance, indicating imatinib as the appropriate definitive treatment in these conditions. The most common cytogenetic alteration in myeloid and lymphoid neoplasms accompanied by eosinophilia and abnormalities of *PDGFRA* is *FIP1L1–PDGFRA* gene fusion first described by Cools *et al.* in 2003 [63].

Table 16. *Schematic overview of 2008 WHO classification of eosinophilic disorders [64]*

Myeloid and lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB*, or *FGFR1*
Diagnostic criteria of an MPN with eosinophilia associated with *FIP1L1–PDGFRA* Diagnostic criteria of MPN associated with *ETV6–PDGFRB* fusion gene or other rearrangemer of *PDGFRB* Diagnostic criteria of MPN or acute leukaemia associated with *FGFR1* rearrangement
Chronic eosinophilic leukaemia, not otherwise specified (CEL-NOS)
Idiopathic hypereosinophilic syndrome (iHES)
Exclusion of the following: 1. Reactive eosinophilia 2. Lymphocyte-variant hypereosinophilia (cytokine-producing, immunophenotypically aberran T-cell population) 3. Chronic eosinophilic leukaemia, NOS 4. WHO-defined myeloid malignancies-associated eosinophilia (e.g. MDS, MPNs, MDS/MPN or AML) 5. Eosinophilia-associated MPNs or AML/ALL with rearrangements of *PDGFRA*, *PDGFRB*, o *FGR1* 6. The absolute eosinophil count (AEC) >1,500/mm^3 must persist for at least 6 months and tissue damage must be present. If there is no tissue damage, iHES is the preferred diagnosis.

Laboratory evaluation of primary hypereosinophilia should start with screening of peripheral blood or bone marrow for *FIP1L1–PDGFRA* gene fusion either by real-time PCR (RT-PCR) or interphase/metaphase fluorescence *in situ* hybridization (FISH). Abnormalities of *PDGFRB* or *FGFR1* could be identified by conventional cytogenetic analysis or FISH [62]. Studies conducted in developed countries indicate that *FIP1L1–PDGFRA* fusion occurs in approximately 10% of patients with hypereosinophilia of unknown aetiology [65-67]. While imatinib mesylate (imatinib) administered in lower doses than in CML and as first-line treatment for patients with abnormalities of *PDGFRA/B* is highly effective to reach and maintain molecular remission, patients with *FGFR1* fusions are resistant to this drug and have a poor prognosis [62].

The WHO definition of HES is based on the historical criteria outlined by Chusid *et al.* in 1975. According to these, the diagnosis of HES can be established if AEC >1,500/mm^3 persists for >6 months and tissue damage is present without secondary causes of eosinophilia (such as parasitic infection, drugs, connective tissue disorders, vasculitis, malignancies, or allergies) [68]. However, the criterion of a 6-month-long duration of elevated AEC may be abandoned as the primary goal is the rapid correction of hypereosinophilia and the prevention of organ failure by an early and effective treatment. In the absence of organ damage, the preferred term is “idiopathic hypereosinophilia” instead of HES [69]. The proper classification is fairly difficult as, on the one hand, there is a considerable overlapping between the various conditions with hypereosinophilia and, on the other hand, the nomenclature itself has been continuously debated for decades.

The most common signs and symptoms in HES are asthenia and fatigue (26%), cough (24%), dyspnoea (16%), myalgia or angiooedema (14%), rash or fever (12%), and rhinitis (10%) [61]. Typical laboratory findings include leukocytosis (≥20,000-30,000/mm^3) with peripheral eosinophilia up to a level of 30-70% [70]. The clinical manifestation of HES is diverse ranging from an asymptomatic form to a progressive course with severe symptoms and multi-organ involvement. Sustained eosinophilia may affect all tissues and organ systems. Most frequently reported complications include dermatological, neurological, pulmonary, cardiac, and – less commonly – gastrointestinal conditions [70-73].

The major cause of morbidity and mortality is eosinophilic myocarditis developing secondary to endomyocardial fibrosis and restrictive cardiomyopathy. Loeffler’s endocarditis with eosinophilic infiltration represents the prototype of cardiac manifestations in HES [7]. The clinical course of Loeffler’s endocarditis typically progresses in three – necrotic, thrombotic,

and fibrotic – stages into endomyocardial fibrosis and restrictive endomyocardyopathy as end-stage [74]. Many hypotheses have been proposed to explain the pathomechanism of cardiac and other organ dysfunctions in HES. The primary target of the tissue damage initiating local thrombosis is endothelial cells of the endocardium [48]. Major basic protein released from eosinophilic granules may exert endothelial cell damage while eosinophilic cationic protein may be responsible for the hypercoagulable state. The direct toxic and procoagulant effect of eosinophilic derivatives may contribute to the development of thrombosis and cardiac embolism [58-61, 71]This may explain central nervous system (CNS) injury: not only eosinophil-induced endothelial dysfunction but also cardiac microembolization might play an important role in the development of cerebral infarction and neurological dysfunction. Toxic effects of released eosinophilic basic proteins may initiate endomyocardial necrosis which occurs in the early stages and usually remains subclinical. After 4-6 weeks of disease onset the excessive release of tissue factor from damaged tissue cells and eosinophils may lead to endomyocardial fibrosis [59, 75-77]. Case series published after the first description of the disease reported very poor prognosis and short survival in HES, primarily due to the advanced state of the disease and the congestive heart failure. In a report by Chusid *et al.*, median survival was as short as 9 months while 3-year survival was only 12% [68]. A later publication on 40 HES patients talked about a 5-year survival rate of 80%, decreasing to 42% at 15 years [73].

3.3.2 PATIENTS AND DATA COLLECTION IN THE HYPEREOSINOPHILIC SYNDROME POPULATION

The diagnosis of idiopathic HES can only be established after the exclusion of all primary and secondary causes of hypereosinophilia and lymphocyte-variant hypereosinophilia [69]. We evaluated 10 iHES patients with hypereosinophilia fulfilling the 2008 WHO criteria but without any secondary causes or underlying clonal disease. All patients were asymptomatic; none of them had a known Loeffler's endocarditis. Cytogenetic, FISH, and molecular analyses of *FIP1L1–PDGFRA* were negative for all subjects in this population of 10 iHES patients without any underlying disease. Due to the presence of Loeffler's endocarditis and the rare neurological complication, the case of the patient with *FIP1L1–PDGFRA* mutation will be discussed separately.

Table 17. *Summary of the main demographic and disease-related characteristics of the investigated study population with hypereosinophilic syndrome*

Characteristics	HES
Main demographic characteristics	
Patients (N)	10
Males (N)	7
Females (N)	3
Median age at diagnosis (years) (range)	58.1 ± 13.1
Median follow-up (months) (range)	37 (2–120)
Disease-related characteristics	
Red blood cell count (T/L)	4.2 ± 0.5
Haemoglobin (g/L)	126.7 ± 18.8
Haematocrit (%)	36.9 ± 5.5
Platelet count (G/L)	276.3 ± 176.7
White blood cell count (G/L)	16.7 ± 5.8
Percentage of blood eosinophils (%)	49.0 ± 16.6
Absolute eosinophil count (G/L)	8.7 ± 4.8

3.3.3 STATISTICAL ANALYSES

All HES patients and their age- and sex-matched healthy controls underwent complete two-dimensional (2D) Doppler echocardiography and 3DSTE. Data of altogether 10 HES patients were compared to matching data of 19 control subjects.

Continuous variables were calculated as mean ± standard deviation. All statistical tests were two-sided. The cut-off value for statistical significance was set at p=0.05. Continuous parameters were compared using unpaired Student's *t* test while categorical variables were analyzed by chi-square or Fischer's exact test. The correlation was defined by Pearson's correlation coefficient. Statistical evaluations were performed using MedCalc software (MedCalc Inc., Mariakerke, Belgium).

3.3.4 METHODS – LABORATORY TESTS AND ECHOCARDIOGRAPHY

Diagnostic bone marrow samples of all patients investigated for hypereosinophilia were evaluated at our local tumour pathology laboratory. Cytogenetic tests were completed at the laboratory for cytogenetics of the 2nd Department of Internal Medicine and Cardiology Centre while FISH assays were performed at the Department of Haematology and Stem Cell Transplantation, United Szent István and Szent László Hospital, Budapest. Molecular genetic tests for *FIP1L1–PDGFRA* were carried out in a close co-operation with the team of the Laboratory of Molecular Diagnostics, Hungarian National Blood Transfusion Service, Budapest [63].

Three-dimensional speckle-tracking echocardiography (3DSTE)

3DSTE datasets were acquired from apical window using the 1–4 MHz matrix phased-array transducer (PST-25SX) [78].

Following gain setting optimization, full volume mode was used over six consecutive cardiac cycles during a single breath-hold. Volume data were stored in raw data format for further analysis. LA quantifications were performed using the Artida 3D Wall Motion Tracking software v2.7 (Toshiba Medical Systems, Tokyo, Japan). Each three-dimensional (3D) dataset was displayed in multiple plane views including the apical two- (AP2CH) and four-chamber (AP4CH) views and three short-axis views at different LA levels from the base to the apex. Several reference points on the LA endocardium were set by the examiner in the AP2CH and AP4CH views. The first points were set at the edge of the septal mitral valve ring where anterior mitral leaflet origins and then markers were placed in a counterclockwise rotation around the LA to the lateral mitral valve ring (to the origin of the posterior leaflet) in the AP4CH viewing plane. During evaluations, LA appendage and the pulmonary veins were excluded from the LA cavity. Measurements were performed first on AP4CH view and then on AP2CH view. After detection of the LA myocardial borders at the end-diastolic reference frame, the user could correct the LA shape if it was necessary. The 3D wall motion tracking was then automatically performed through the entire cardiac cycle.

3DSTE for left atrial volumetric measurements

From time curves of global LA volume changes, maximum (V_{max}), minimum LA volumes (V_{min}) and LA volume before atrial contraction (V_{preA}) were measured using the 3D echocardiographic datasets just before mitral valve opening (end-systole), just before mitral valve closure (end-diastole) and at time of P-wave on electrocardiography (early diastole), respectively [78-82] LA function consists of three phases: the systolic reservoir phase and the diastolic passive (conduit) and active emptying (booster pump) phases. To characterize these functions, stroke volumes (SVs) and emptying fractions (EFs) were calculated from the above-mentioned volumes as follows:

Left atrial stroke volumes

- Total Atrial Stroke Volume (TASV): $V_{max}-V_{min}$ (reservoir function)
- Passive Atrial Stroke Volume (PASV): $V_{max}-V_{preA}$ (conduit function)
- Active Atrial Stroke Volume (AASV): $V_{pre\ A}-V_{min}$ (booster pump/active contraction function)

Left atrial emptying fractions

- Total Atrial Emptying Fraction (TAEF): $TASV/V_{max}\times100$ (reservoir function)
- Passive Atrial Emptying Fraction (PAEF): $PASV/V_{max}\times100$ (conduit function)
- Active Atrial Emptying Fraction (AAEF): $AASV/V_{preA}\times100$ (booster pump/active contraction function)

3DSTE for left atrial strain measurements

From the same 3D echocardiographic datasets, time curves of unidirectional radial (RS), longitudinal (LS), and circumferential strain (CS) were also generated for each segment using the 16-segment model obtained for LV [81-84] Moreover, due to the ability of 3DSTE to calculate complex strains, area strain (the ratio of endocardial area change during cardiac cycle) and 3D strain (strain in the direction of wall thickening; combination of "unidirectional" strains) were also measured. At each time, segmental strain curve peak strains representing characteristics of reservoir phase of the LA function were measured. Global strains were calculated by the software considering the whole LA while mean segmental strains were obtained as the average of strains of 16 segments as well. These parameters were calculated automatically by the software.

3.3.5 *RESULTS*

COMPARISON OF LEFT ATRIAL VOLUMETRIC, VOLUME-BASED FUNCTIONAL, AND STRAIN PARAMETERS OBTAINED BY THREE-DIMENSIONAL SPECKLE-TRACKING ECHOCARDIOGRAPHY, A NOVEL, NON-INVASIVE CLINICAL TOOL FOR VOLUMETRIC AND STRAIN ANALYSIS, BETWEEN PATIENTS WITH HYPEREOSINOPHILIC SYNDROME AND MATCHED CONTROLS (*Aim III.a*)

On routine haematological testing, the following results (HES vs. controls) were obtained: RBC: 4.2 ± 0.5 T/L vs. 4.3 ± 0.4 T/L (p=0.94), haemoglobin: 126.7 ± 18.8 g/L vs. 130.1 ± 10.2 g/L (p=0.86), PLT: 276.3 ± 176.7 G/L vs. 282.4 ± 158.2 G/L, Htc: 36.9 ± 5.5% vs. 37.8 ± 4.9%, WBC: 16.7 ± 5.8 G/L vs. 6.8 ± 1.2 G/L (p=0.02), eosinophil ratio: 49.0 ± 16.6% vs. 3.2 ± 2.3% (p=0.001), and AEC: 8.7 ± 4.8 G/L vs. 0.4 ± 0.1 G/L (p=0.001). No correlation was found between any of the laboratory findings and 2D echocardiographic or 3DSTE data in this patient population. None of the control and HES patients exhibited >Grade 1 mitral or tricuspid regurgitation. Significant difference was only found in LA diameter and interventricular septum thickness between HES and control subjects. Clinical data of each patient with organic involvement are presented in Table 18. Only one patient had a prior cardiac disease (NSTEMI) in his history.

Table 18. *Clinical data of patients with hypereosinophilic syndrome*

CASE NO. AGE/SEX/DATE OF DIAGNOSIS	ORGANIC INVOLVEMENT	HEPATOMEGALY, SPLENOMEGALY	CARDIAC DISEASE (YEAR)
Case 1 52/M/2013	duodenal eosinophilia	splenomegaly	–
Case 2 71/M/2009	–	–	–
Case 3 44/M/2010	–	–	–
Case 4 66/F/2011	tissue eosinophilia	–	–
Case 5 77/M/2013	–	–	NSTEMI (2013)
Case 6 69/F/2009	eosinophil dermatitis	–	–
Case 7 45/M/2011	sensoral motoneuritis, pulmonal affection, sural necrotising granulomatous vasculitis	splenomegaly	–
Case 8 59/M/2013	–	–	–
Case 9 41/F/2002	–	–	–
Case 10 73/F/2014	bronchial asthma, vasculitis	–	–

NSTEMI, non–ST-segment elevation myocardial infarction

As reflected by 3DSTE data, both global and mean segmental peak CS were significantly reduced in HES patients suggesting an impaired LA reservoir function (Table 19).

Table 19. *Comparison of 3DSTE-derived global and mean peak segmental strain parameters of patients with hypereosinophilic syndrome and controls*

	HES patients (N=10)	Controls (N=19)	p-value
Global strain parameters			
Radial strain (%)	-17.7 ± 7.7	-15.7 ± 11.6	0.64
Circumferential strain (%)	18.3 ± 6.7	25.6 ± 9.0	0.03
Longitudinal strain (%)	21.0 ± 6.2	22.3 ± 8.7	0.68
3D strain (%)	-10.1 ± 5.0	-9.3 ± 9.0	0.81
Area strain (%)	41.2 ± 13.8	50.7 ± 20.4	0.20
Mean segmental strain parameters			
Radial strain (%)	-20.6 ± 6.1	-19.5 ± 8.1	0.70
Circumferential strain (%)	22.2 ± 6.0	31.0 ± 12.1	0.04
Longitudinal strain (%)	21.8 ± 6.4	25.6 ± 7.5	0.18
3D strain (%)	-14.7 ± 4.3	-13.7 ± 6.7	0.67
Area strain (%)	45.6 ± 13.1	58.3 ± 21.7	0.10

3D, three-dimensional; HES, hypereosinophilic syndrome.

Significantly increased maximum (p=0.01) and minimum (p=0.03) LA volumes as well as LA volume before atrial contraction (p=0.01) and elevated total (p=0.02) and active (p=0.005) atrial SVs values characterizing reservoir and booster pump LA function were found in HES patients, as compared to controls. EF did not significantly differ between groups (Table 20).

Table 20. *Comparison of 3DSTE-derived volumetric and volume-based functional left atrial parameters of patients with hypereosinophilic syndrome and controls*

	HES patients (N=10)	Controls (N=19)	p-value
Calculated volumes			
Maximum LA volume ($V_{max,}$ mL)	72.9 ± 38.8	45.6 ± 15.5	0.01
V_{max} / BSA (mL/m^2)	41.8 ± 25.0	26.0 ± 9.7	0.03
V_{max} / BMI [mL/(kg/m^2)]	2.8 ± 1.5	1.8 ± 0.6	0.03
Minimum LA volume ($V_{min,}$ mL)	46.3 ± 33.3	26.0 ± 15.0	0.03
V_{min} / BSA (mL/m^2)	26.8 ± 21.4	14.9 ± 9.7	0.05
V_{min} / BMI [mL/(kg/m^2)]	1.7 ± 1.2	1.0 ± 0.6	0.05
LA volume before atrial contraction ($V_{preA,}$ mL)	62.0 ± 36.0	36.5 ± 16.6	0.01
V_{preA} / BSA (mL/m^2)	35.7 ± 23.1	20.9 ± 10.4	0.03
V_{preA} / BMI [mL/(kg/m^2)]	2.3 ± 1.4	1.4 ± 0.7	0.03
Stroke volumes (SVs)			
Total atrial SV (TASV, mL)	26.6 ± 8.5	19.6 ± 6.4	0.02
TASV / BSA (mL/m^2)	15.0 ± 4.9	11.1 ± 3.6	0.04
TASV / BMI [mL/(kg/m^2)]	1.0 ± 0.3	0.8 ± 0.3	0.12
Passive atrial SV (PASV, mL)	10.9 ± 8.2	9.1 ± 5.0	0.47
PASV / BSA (mL/m^2)	6.1 ± 4.6	5.1 ± 2.8	0.61
PASV / BMI [mL/(kg/m^2)]	0.4 ± 0.3	0.4 ± 0.2	0.78
Active atrial SV (AASV, mL)	15.7 ± 5.1	10.5 ± 4.0	0.005
AASV / BSA (mL/m^2)	8.9 ± 2.9	5.9 ± 2.3	0.01
AASV / BMI [kg/(mL/m^2)]	0.6 ± 0.2	0.4 ± 0.2	0.03
Emptying fractions (EFs) (%)			
Total atrial EF	40.0 ± 10.5	45.0 ± 12.9	0.29
Passive atrial EF	15.9 ± 11.7	21.4 ± 10.8	0.21
Active atrial EF	28.6 ± 7.8	30.5 ± 9.5	0.58

BMI, body mass index; BSA, body surface area; HES, hypereosinophilic syndrome; LA, left atrial.

PRESENTATION OF LEFT VENTRICULAR ROTATIONAL MECHANICS THROUGH A UNIQUE CASE OF HYPEREOSINOPHILIC SYNDROME WITH LOEFFLER'S ENDOCARDITIS BY MEANS OF THE NOVEL METHOD OF 3DSTE (*Aim III.b*) [85]

Loeffler's disease is associated with stiffened ventricular and atrial walls leading to inadequate filling, decreased preload, diastolic dysfunction, and heart failure. At this moment, little is known about the rotational characteristics of the Loeffler's heart. Therefore, we present a 36-year-old male patient with known Loeffler's endocarditis on optimal therapy whose rotational parameters were evaluated by 3DSTE. The multiple long- (A, B) and short-axis views (C3, C5, C7) extracted from a 3D echocardiographic dataset are shown in Figure 6. Visual information on LV rotation is given in colour overlay superimposed on grey-scale images. Moreover, a 3D cast of LV (Figure 6D) and calculated LV volumes, ejection fraction (EF), and estimated LV mass are also demonstrated (Figure 6E). Quantitative data are provided on LV rotation with adequate rotational directions with counterclockwise motion of the LV apex (white arrow, positive value) and clockwise motion of the LV base (dashed arrow, negative value) (Figure 6F). Both LV apical and basal rotation were in normal range suggesting normal rotational characteristics despite reduced systolic function in this case with known Loeffler's disease. Data of a control subject are also presented in the same fashion (Figure 7) [85].

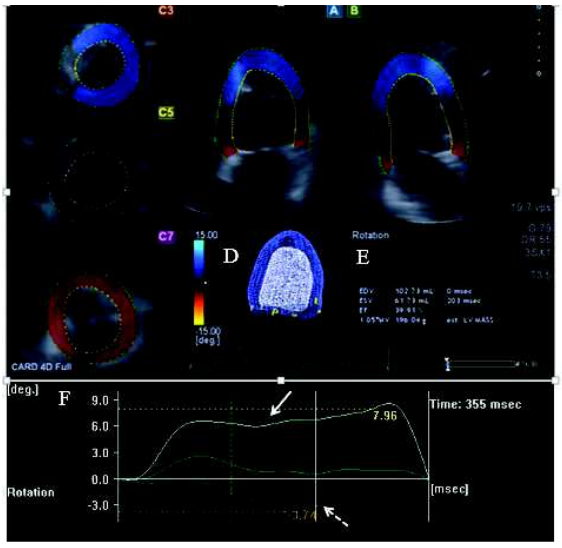

Figure 6. *Apical four-chamber (A) and two-chamber (B) views and short-axis views (C3, C5, C7) at different levels of the left ventricle (LV) extracted from the three-dimensional (3D) echocardiographic dataset. A 3D cast of the LV (D) and calculated volumetric and functional LV parameters (EDV, end-diastolic volume; ESV, end-systolic volume; EF, ejection fraction; est LV MASS, estimated left ventricular mass) are also demonstrated (E). Counterclockwise rotation of LV apex (white arrow, positive value) and clockwise rotation of LV base (dashed arrow, negative value) are also shown demonstrating normal rotational directions in this Loeffler patient's heart (F).*

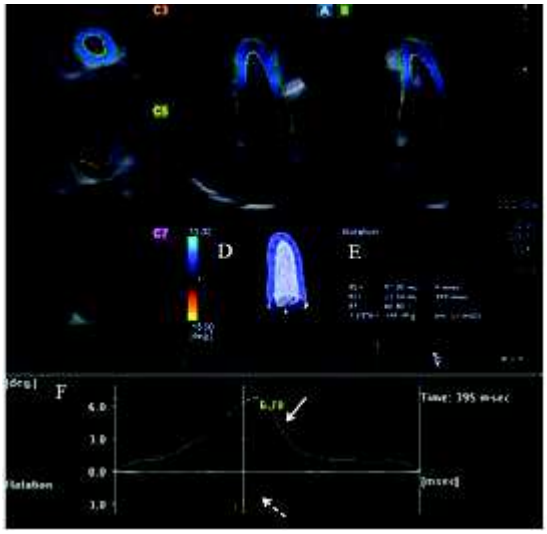

Figure 7. *Apical four-chamber (A) and two-chamber (B) views and short-axis views (C3, C5, C7) at different levels of the left ventricle (LV) extracted from the three-dimensional (3D) echocardiographic dataset. A 3D cast of the LV (D) and calculated volumetric and functional LV parameters (EDV, end-diastolic volume; ESV, end-systolic volume; EF, ejection fraction; est LV MASS, estimated left ventricular mass) are also demonstrated (E). Counterclockwise rotation of LV apex (white arrow, positive value) and clockwise rotation of LV base (dashed arrow, negative value) are also shown demonstrating normal rotational directions in a healthy subject (F).*

Moreover, it is important to emphasize that our research identified an uncommon case in the investigated patient population. Due to its unique nature and potential clinical relevance, we demonstrated it in a separate case report, providing therefore an additional proof of the complexity of HES [86]. The case is presented in details in *Appendix VI* to this dissertation as the publication of Imelda Marton *et al.*: *Watershed infarction in hypereosinophilic syndrome: a diagnostic dilemma in FIP1L1–PDGFR-alpha-associated myeloid neoplasm and overview of the relevant literature* [86].

4 DISCUSSION

4.1 POLYCYTHAEMIA VERA

PV, the most common *BCR–ABL1*-negative classic MPN is a HSC-derived clonal myeloproliferation characterized by a trilineage expansion of morphologically normal red cells, white cells, and platelets without significant bone marrow fibrosis. At the 2nd Department of Internal Medicine and Cardiology Centre, which is a regional haematological centre for the population of south-eastern Hungary, data on 108 PV patients were available from the period between 1998 and 2014. As reported [10], the incidence of PV varies from 0.4 to 2.8 cases per 100,000 persons per year in the European Union; the median age at diagnosis is around 61 years (range: 18–95 years) [11]. The occurrence of PV is a bit higher in men than in women (2.8 vs. 1.3 cases per 100,000 persons per year [12]. In our

study, demographics like distribution by gender and mean age at diagnosis were highly consistent with corresponding data in the literature.

The diagnostic criteria of PV have been changed several times since the first edition of the PVSG criteria in 1975. The identification of *JAK* mutation fundamentally affected the diagnosis of PV. The acquired *JAK2 V617F* somatic mutation causes cytokine-independent activation of several biochemical pathways involved in the erythropoietin receptor signalling. Given that more than 90% of PV patients carry this particular mutation, it has been incorporated into the 2008 WHO diagnostic criteria. The lack of clear diagnostic criteria in the past often resulted a proportion of secondary polyglobulia cases to be diagnosed and followed as PV. Therefore, we strictly adhered to the 2008 WHO criteria while establishing our PV database and excluded all polyglobulia cases which did not meet these criteria (i.e., cases without *JAK2* mutation, combined with normal or increased serum erythropoietin levels). The proportion of PV patients with *JAK2 V617F* mutation in our PV database was 94.4% which correlates well with the existing literature [17]. The incidence of *JAK2* exon 12 mutation (which has a reported frequency of 3%) was not investigated in our research.While earlier studies estimated the rate of vascular events to 12–39% and reported about various manifestation forms [18], recent works have also confirmed their incidence well above 20% [17]. Arterial events occur typically much more frequent than venous ones [20].

In PV, thrombosis (splanchnic venous thrombosis or cerebral sinus thrombosis) is often an initial occurrence which may suggest the diagnosis as early as before the haematological verification. In our study, the prediagnostic incidence of major thrombotic events was 33/108 (30,5%), highly similar to literature data [87]. Another research found the frequency of pre- and peridiagnostic major vascular events as 16% for arterial and 7% for venous complications, respectively, while the incidence of haemorrhagic events made up 4% [88]. In the largest prospective European PV study (European Collaboration Study on Low-dose Aspirin in Polycythemia – ECLAP, 2004), the same incidence rates accounted for 27%, 11%, and 9.2%, respectively [89]. Later investigations like the CYTO-PV trial (2013) reported 17% and 12% for the incidence of arterial and venous complications, respectively, while these numbers were 12% and 9% in another study conducted in 1545 patients by Tefferi *et al.* (2013) [11, 88, 90]. Decreasing incidence rates might be explained by the evolution of diagnostic criteria and the incorporation of novel knowledge into the therapeutic regimens (e.g., based on results from the ECLAP study, wider use of ASA, more aggressive phlebotomy policy for more restrictive Hct target, introduction of cytoreductive treatment, and more intensive management of CV risk factors).

In our PV patient cohort representing the period from 1998 to 2014, a total of 20 major vascular complications were observed in 108 patients (18.5%); among them, arterial events (n=18; 16.6%) occurred more frequently then venous ones (n=2; 1.8%). The frequency of total vascular and arterial events in our research was similar to those in the above trials, with consistently lower rates for venous thrombotic events. Furthermore, we thoroughly investigated the incidence of CV events during follow-up and found them to occur in 7 cases in 108 patients (6%). This means, six out of seven PV patients were available for an adequate cardiological analysis of CV complications during follow-up. All patients belonged to the high-risk group and five of them (83%) had at least two or more conventional CV risk factors. The frequency of AMI in our PV cohort was higher (6%) than that reported in trials on larger patient populations (2%) [88].

Compared to the literature (7%), we detected a markedly higher frequency for cerebrovascular events (11/108; 10.1%) [88]. The occurrence of cerebrovascular complications in our PV cohort also exceeded the incidence of stroke reported earlier in the general Hungarian population (2/1,000 in the age of 45–54 years, 3/1,000 in the age of 55–64 years, and 3–13/1,000 above the age of 65 years; 2005) [91]. In most cases, chronic ischaemic white matter lesions were observed. Mild cerebral atrophy was also a frequent finding. The clinical presentation of cerebrovascular events was predominated by lacunar syndromes or VBI. Most of these patients (8/11; 72%) also presented at least two major conventional CV risk factors. The slightly higher AMI and stroke incidence rates in our PV cohort might be attributed to our less stringent – yet historically guideline-compliant – attitude in terms of Hct targets and the introduction of cytoreductive treatment at that time; i.e., our treatment strategy used to be based on the conventional two-level risk assessment. In addition, the significant additional effect of CV risk factors on thrombotic events was only partially acknowledged and this approach in PV management was therefore much less dominant.

Although CV risk factors were incorporated into the IPSET score in 2012 for purposes of thrombosis risk stratification in ET, the same practice is still disputed for PV [92]. The results of our research in PV in terms of the incidence of CV and cerebrovascular complications and the contribution of CV risk factors to subsequent thrombotic complications clearly demonstrate the importance of considering PV as a prothrombotic state where conventional CV risk factors (hypertension, tobacco use, hyperlipidaemia, obesity, DM) may significantly increase the risk of thrombotic events. The complete pathomechanism of prothrombotic processes in PV remains, however, to be understood. Falanga *et al.* carried out intensive investigations on the correlation between MPNs and thromboses and found the

pathogenesis to be particularly complex and multifactorial. The higher thrombosis risk in PV can be considered as a result of as a consequence of an acquired thrombophilic state associated with the underlying disease. This might be partly explained by the prothrombotic characteristics of MPN clone-derived blood cells and the procoagulant response of normal epithel cells to inflammatory stimuli. In addition, higher Hct rates result in blood hyperviscosity. The greater red cell mass displaces platelets toward the vessel wall, thus facilitating shear-induced platelet activation and aggregation and enhancing platelet-platelet interaction. Moreover, changes of the red cell membrane trigger the formation of erythrocyte aggregates. Finally, all these processes together are responsible for the increased coagulability of blood in PV [20]. Although the recommended Hct level was questioned for a long time, [93], current gudelines clearly indicate a target of 45% in PV [17, 21, 90]. Our observation that the frequency of thrombotic events during follow-up differed significantly between PV subgroups with Hct values below or above 45% is well reflected by the latest therapeutic recommendations.

Currently, no data are available whether the presence of one CV risk factor or two or more CV risk factors considerably increases the occurrence of thrombotic complications in PV. Our research demonstrated that there is a significant difference in the thrombosis-free survival between PV patients with or without at least one CV risk factor. The difference is also significant for the comparison of PV patients with at most one CV risk factor or at least two CV risk factors. These results were further confirmed by our study to evaluate the role of CV risk factors in major thrombotic complications in the pooled population of female patients with ET or PV. In this setting, the presence of two or more CV risk factors was associated with a significantly higher risk of thrombosis. Moreover, a significant difference was seen in the thrombosis-free survival between patients with at most one CV risk factor and those with two or more CV risk factors [25]. Female patients with CV risk factors and PV or ET may well be at a higher risk of thrombotic events, and require therefore a special consideration for the prevention and management of thrombotic events.

Regarding thrombotic events, the evaluation of additional risk factors is a novel approach. The first papers emphasizing the role of CV risk factors in addition to conventional risk assessment (age >60 years and/or prior thrombotic event) were published in the last year of our research. Moreover, the 2015 update on PV management by Tefferi *et al*. implementing new results provides much more complex recommendations than the previous versions used to be based on a two-level (low or high) risk assessment [17]. Although CV risk factors were not incorporated in the risk stratification of the 2015 guidelines of the European Society for

Medical Oncology, they are taken into account in their risk-adapted therapy recommendations [94]. Given that in PV, thrombohaemorrhagic events represent the leading cause of morbidity and mortality, the therapy primarily aims to prevent these complications without increasing the bleeding risk, and only secondarily to control the symptoms. Therefore, treatment should always be individually adjusted to thrombosis and/or bleeding risk. Knowledge is being continuously updated and implemented in the guidelines, resulting in even more stringent therapeutic recommendations. Controlled trials showed the reduction of thrombosis risk with low-dose ASA and cytoreductive therapy in high-risk patients, without affecting the survival [89, 90]. Our results support the importance of individualized treatment – considering each patient's thrombosis and/or bleeding risk – and more aggressive management of modifiable risk factors (e.g. CV risk factors) with restrictive targets in PV.

Patients defined as being at low risk according to previous therapeutic guidelines (age <60 years, without prior thrombotic event) used to be treated with anti-platelet agents (e.g. ASA) and by phlebotomy, while high-risk patients (age ≥60 years and/or with prior thrombotic event) used to receive cytoreductive drugs (e.g. hydroxyurea) combined with anti-platelet therapy. Current therapeutic recommendations for PV are largely adapted to individual risk profile. For low-risk patients (age <60 years and negative thrombosis history and the presence of *JAK2 V617F* mutation or CV risk factor(s)) without extreme thrombocytosis, low-dose ASA once daily is recommended; in case of CV risk factors with concomitant *JAK2 V617F* mutation positivity ASA twice daily should be considered. In low-risk patients with extreme thrombocytosis (PLT >1,000×10^9/L), the measurement of ristocetin cofactor activity is necessary (administration of ASA if >30%). High-risk patients (age ≥60 years and/or positive thrombotic history) without prior thrombotic events should be treated with hydroxyurea + once daily low-dose ASA. In case of previous arterial thrombotic event, hydroxyurea + ASA should be given, with the consideration of ASA twice daily if any or more of the following are present (CV risk factor(s), *JAK2 V617F* mutation positivity, age >60 years). For high-risk patients with previous venous event, hydroxyurea with life-long anticoagulation is recommended, given the concept of PV as an acquired persistent thrombophilic state. The addition of ASA once daily should also be considered if any or more of the following are present (CV risk factor(s), *JAK2 V617F* mutation positivity, age >60 years). In all patients, pharmacological therapy should always be completed by phlebotomy to target Hct <45% [17, 95]. For high-risk patients refractory to or intolerant of hydroxyurea, interferon-alpha (IFN-alpha; age <65 years) or busulphan (age >65 years) may be an option. The selective JAK1/2 inhibitor ruxolitinib approved in 2015 for the treatment of

adult patients with PV who are resistant to or intolerant of hydroxyurea is not yet widely available in Hungary.

4.2 SYSTEMIC MASTOCYTOSIS

Mastocytosis is an orphan disease characterized by the clonal neoplastic proliferation of mast cells accumulating in one or more organ systems. Out of the several manifestations of adult mastocytoses, our research targeted adult SM. According to the 2008 WHO criteria, SM is classified as ISM, SM-AHNMD, ASM, and MCL. In our regional centre, 14 ISM, 15 AHNMD, and 6 ASM cases were diagnosed in the period between 2001 and 2013; MCL was, however, not detected in any patient. As our study aimed at the evaluation of histological features, molecular characteristics as well as laboratory and clinical parameters of adult SM, mastocytosis cases in paediatric patients or those in adults confined only to the skin (without available bone marrow biopsy results) were not included in our research. All bone marrow test results since 2001 were screened for SM and re-assessed by morphological, immunohistochemical, and molecular (PCR) methods, according to the 2008 WHO criteria.

ISM is the most frequent subtype of SM in adults. It is predominated by cutaneous manifestations (UP) but recurrent systemic symptoms related to mast cell degranulation and mediator release and/or allergies or anaphylaxis may also occur. A wide range of factors may lead to mast cell activation, resulting in severe or even life-threatening or fatal symptoms [26, 38]. In our SM study population, cutaneous manifestation (UP) was detected in 57% (8/14) of the patients while mediator-related symptoms occurred in 28% (4/14) of them.

In our study, the main cause of the indication of bone marrow biopsy in the group of ISM patients was UP (8/14; 57%). It is important therefore that dermatologists should refer any patient with CM lesions to a haematological centre to check potential bone marrow involvement. In other cases, bone marrow biopsy was performed for eosinophilia (3/14; 21%), anaemia (1/14; 7%), lytic bone lesions (1/14; 7%), or adenomegaly (1/14; 7%).

On the contrary, symptoms in ASM (e.g. cytopenia, ascites, malabsorption, or osteolytic skeletal lesions) result from organ dysfunction due to mast cell infiltration. In the entire ASM subgroup (6/6; 100%), bone marrow biopsy was requested due to either anaemia with or without thrombocytopenia or hepatosplenomegaly with or without constitutional symptoms [55]. All ASM patients presented with at least one C finding, as defined by the 2008 WHO criteria (marked cytopenia, osteolysis with or without pathologic fractures, ascites and elevated liver enzymes, malabsorption with hypalbuminaemia, palpable splenomegaly with hypersplenism).

In the SM-AHNMD subgroup, mainly symptoms of the associated neoplasm indicated bone marrow biopsy. In most patients (14/15; 93%), these were symptoms of pancytopenia, anaemia, leukocytosis, thrombocytosis, bone pain/lesion, or hepatosplenomegaly while in one case, the associated SM was revealed by concurrent lymphoma staging process. Regarding associated clonal diseases, our findings were consistent with previous reports [27]. Also in our study, primarily myeloid neoplasms were identified (12/15; 80%) while lymphoid neoplasms were much less frequently detected (3/15; 20%). As provided in current literature [46], we found ISM to be mainly accompanied by cutaneous symptoms (8/14; 57%); however, we observed a lower rate of this association in ASM (2/6; 33%) and the lack of such manifestation in SM-AHNMD.

Most of the adult patients with mastocytosis, regardless of disease subtype, are positive for the somatic activating mutation of the oncogenic receptor tyrosine kinase *KIT* (exon 17, D816V) [33, 42-45] The *KIT D816V* mutation, which is found in up to 85% of all SM patients, is of both pathogenetic and diagnostic relevance [26, 43-45].

In our SM patient population, *C-KIT* mutations detected by PCR were confirmed by Sanger sequencing. Out of all detected cases, only those were enrolled in the SM study which strictly met the relevant 2008 WHO criteria (major criterion and ≥1 minor criterion, OR ≥3 minor criteria). In accordance with literature data, *KIT D816V* mutation positivity was detected in 78% (11/14) of ISM patients, 80% (12/15) of AHNMD patients, and 83% (5/6) of ASM patients, respectively [31, 96].

The clinical presentation of SM is highly heterogeneous ranging from asymptomatic manifestations (ISM) to severe, progressive disease forms greatly affecting life expectancy (ASM, MCL) [31]. The correct diagnosis is therefore of crucial importance, combining the analysis of histopathological and molecular characteristics with the assessment of B and C findings. The complex evaluation of these parameters will determine which WHO subcategory each patient is to be assigned to. The updated 2008 WHO nomenclature with its strong prognostic implications provides a much better prediction of the typical clinical course and the expected survival in each SM subtype. A long-term Spanish study revealed that the great majority of adult ISM patients are able to live a normal life [97].

The results of the ever largest trial to confirm the prognostic value of the current WHO classification system were published in 2009 [27]. Within this study, clinical, laboratory, and survival data of 342 adult patients diagnosed with SM over a 30-year-long period were recorded and evaluated. Compared to the non-indolent forms of SM (AHNMD and ASM), the life expectancy in the ISM group was considerably higher and not significantly different from

the age- and sex-matched American (USA) population for the appropriate time period, based on the date of diagnosis [27].

The aim of our research was to analyse the set of adult SM cases emerged in a cohesive geographic region of Hungary. The distribution of SM subtypes in our study population of 35 patients was mostly similar to that reported in the largest clinical trial in SM with the updated WHO criteria: ISM 14/35 (40%), SM-AHNMD 15/35 (42%), ASM 6/35 (17%) and MCL 0%, vs. 46%, 40%, 12%, and 1%, respectively [27, 50].

Our analyses on Hungarian patients revealed that the survival in SM (including all subtypes) is worse than that expected in the age- and sex-matched Hungarian population. The life expectancy of patients with ISM was excellent whereas SM-AHNMD and ASM groups had a reduced median survival. The distribution of subtypes and the survival pattern seen in our SM study population was similar to that previously reported in the Mayo Clinic trial [27, 50].

To date, only limited epidemiologic data are available in SM. The prevalence of mastocytosis in Central Europe is estimated at 0.5-1/10,000 [37]. Local/regional data on ISM have been reported from The Netherlands (Groningen) and population-based epidemiological data from Denmark [36, 38, 39]. Moreover, the recently established centralized ECNM registry is still collecting data from ten European countries; therefore, epidemiological data on the prevalence of SM are already available [36]. We found the 13-year cumulative incidence of SM in the general population aged 15 years or more amounts to 0.27/10,000 which meets the criterion of orphan diseases. As in the most relevant literature [37], we could not report a gender predominance. Our data on the cumulative incidence of SM are the first such results published in Hungary. Since only cases that strictly complied with the WHO criteria were included in our SM investigations, the cumulative incidence of SM calculated during our research is likely to be somewhat underestimated. Patients with skin lesions who refused bone marrow biopsy were not enrolled in our analyses. The real cumulative incidence might be higher because SM is often underdiagnosed due to its subtle or even absent symptoms.

Within the SM cohort, we individually reported the course of a female patient with ISM followed-up for 27 years, an unusual case of particular clinical relevance [54]. During this uniquely long follow-up, she experienced recurrent specific cutaneous symptoms which greatly impaired her quality of life. As her cutaneous symptomes showed only slight and temporary improvement on multiple symptomatic treatments (antihistamine, sodium cromoglicate, psoralen + ultraviolet A light [PUVA] therapy, IFN-alpha), imatinib mesylate was introduced even though neither imatinib-resistant *KIT D816V* nor any imatinib-sensitive *KIT* mutation was identified. Unexpectedly, the patient with this *KIT D816V* mutation-

negative disease achieved a considerable reduction of skin lesions and a temporary appreciable improvement of her quality of life on imatinib.

Currently, there is no curative treatment for SM. The available therapies (including histamine receptor antagonists and other antimediator agents) give only symptomatic relief by decreasing the effects of mast cell activation. An important component of SM treatment is the elimination of known symptom triggers. The perioperative management of SM patients is particularly difficult: a multidisciplinary preoperative assessment, an adequate premedication, and a close intra- and postoperative monitoring are of outstanding importantance [28]. Cytoreductive and targeted therapies (tyrosine-kinase inhibitors) can only be considered in aggressive and leukaemic SM variants. As the vast majority of SM cases harbour the known imatinib-resistant *KIT D816V* mutation, currently available tyrosine-kinase inhibitors are, unfortunately, ineffective. Other drugs like multikinase inhibitors are still under clinical investigation. The prognosis of these patients remains poor, even if treated with novel Kit-targeting agents, polychemotherapy, or HSC transplantation [26].

4.3 HYPEREOSINOPHILIC SYNDROME

Cardiac manifestations are the major cause of morbidity in HES and develop in three stages. The first acute, mostly asymptomatic necrotic stage is due to eosinophilic infiltration of the myocardium. The initial damage is thought to be mediated by the contents of the eosinophilic granules. The intermediate (thrombotic) phase is characterized by thrombus formation followed by thrombus organization into a thick layer of granulation tissue. In the third, fibrotic stage, granulation tissue evolves into fibrosis with a small inflammatory zone. Nowadays, the term “Loeffler’s endomyocarditis” is used to describe the thrombotic and fibrotic stage of cardiac involvement in HES. Typical echocardiographic findings include endocardial thickening, fibrothrombotic obliteration of the ventricular apices, and valvular regurgitation due to restricted motion of the posterior mitral leaflet as assessed by routine 2D Doppler echocardiography. At enrolment, the majority of our HES patients did not have any known cardiovascular disease (except for Case 5) or clinical signs of thrombosis/fibrosis characteristic of Loeffler’s endocarditis. They represented theoretically the first – asymptomatic – stage of the disease; therefore, any subsequent alteration in LA morphology and function could be attributed solely to HES. Only LV hypertrophy and dilated LA could be detected by conventional 2D Doppler echocardiography without significant valvular regurgitations or thrombus formation. 3DSTE confirmed LA volumetric changes in all phases of LA function and found alterations in both global and mean peak segmental LA-CS,

suggesting a reduced LA reservoir function and remodelling. Wide spectrum of pathophysiological changes could lead to LA remodelling with structural, functional, or neurohormonal etc. consequences. The real mechanism behind LA remodelling in HES is not completely known, but myocyte necrosis, alterations of the extracellular matrix and in the release of atrial hormones due to toxic proteins from degranulating eosinophils, and diastolic dysfunction could explain our findings.

3DSTE is a new clinical tool for non-invasive 3D cardiac chamber quantification of the LV and LA. The technique is based on the so-called “block-matching algorithm” of the myocardial speckles during their frame-to-frame motion. 3DSTE has been demonstrated to be useful for LA volumetric and strain assessments, allowing more detailed evaluation of LA function from the same 3D dataset. Different patterns in 3DSTE-derived volume-based and strain functional properties could be demonstrated in different disorders. In a recent 3DSTE study, peak LA-RS and LA-LS were found to be altered in hypertrophic cardiomyopathy along with preserved LA-CS. In another study, all strains at all LA levels showed alterations in atrial fibrillation by 3DSTE. In the present study, only peak LA-CS was decreased while RS and LS remained unchanged in HES patients. The pathomechanism of this phenomenon, i.e. only LA-CS showed alterations in HES is unknown, but haemodynamic factors and their relationship with LA fiber orientation could not be excluded, in addition to the above processes [7-9, 79-81].

Our investigations were the first in HES with 3DSTE. Moreover, it is important to emphasize that our research identified an uncommon case. Due to its unique nature and potential clinical relevance, we demonstrated it in a separate case report, providing therefore an additional proof of the complexity of HES. The patient with hyperosinophilia in our report [86] had involvement of the CNS and the myocardium. After the onset of neuropsychiatric symptoms, magnetic resonance imaging indicated bilateral cerebral and cerebellar cortical-subcortical lesions involving the watershed areas, mainly in the parieto-occipital regions. The first transthoracal echocardiography (TTE) during the presence of neurological symptoms did not reveal any pathological findings, but repeated TTE two weeks later suggested the involvement of the myocardium in the form of Loeffler’s endocarditis. Abdominal ultrasonography showed splenomegaly. Clinical data and bone marrow histopathology confirmed the diagnosis of HES. No chromosomal aberration was detected, and subsequent molecular tests by FISH demonstrated *FIP1L1–PDGFRA* gene rearrangement. High-dose intravenous steroid (methylprednisolone 500 mg/day) alleviated the neurological symptoms within a few weeks while low-dose imatinib (200 mg/day) resulted in an impressive

regression of hypereosinophilia and splenomegaly in 6 weeks. The pathogenesis of the neurological dysfunction in HES is potentially explained by a number of hypotheses: a) direct infiltration of eosinophil cells; b) neurotoxic effect of major basic protein and eosinophil cationic protein released from eosinophilic granules; c) local thrombosis due to eosinophil-induced endothelial dysfunction; or d) brain infarction caused by microembolization from endomyocardial fibrosis [98-100]. During follow-up, the patient continued to receive imatinib and experienced persistent remission in a stable condition without developing any new complaints. The diagnostic work-up requires a close and effective multidisciplinary co-operation between the neurologist, the neuroradiologist, cardiologist and the haematologist in order to achieve an early and accurate diagnosis and the successful management of a patient with a *FIP1L1–PDGFRA*-positive myeloid neoplasm.

For patients with strictly defined HES (i.e., after the exclusion of any possible cause of a secondary hypereosinophilia), basic therapy consists of corticosteroids, with or without hydroxyurea, for the rapid reduction of AEC. For steroid non-responders, hydroxyurea may be administered alone. IFN-alpha can induce both haematological and cytogenetic remission in HES and CEL patients refractory to prior therapies. Currently, anti-interleukin-5 or anti-CD52 monoclonal antibody treatment is mainly used as experimental therapy in patients with *PDGFRA/B*-negative HES. Treatment choice may vary substantially depending on the underlying eosinophilic condition (whether a targeted molecular therapy is considered or not) and the extent of organ damage.

5 SUMMARY

CONCLUSIONS AND CLINICAL IMPLICATIONS OF OUR RESEARCH

For purposes of our research focussing especially on Ph-negative MPNs, we retrospectively established a database of PV, SM, and HES cases diagnosed at the 2nd Department of Internal Medicine and Cardiology Centre between 1998 and 2014. The collected data were analyzed and assessed by disease-relevant factors and in accordance with literature requirements.

5.1 POLYCYTHAEMIA VERA

In PV, the most common type of Ph-negative MPDs our research focussed on vascular complications. Vascular events represent the primary cause of morbidity and mortality in PV and require therefore a strong multidisciplinary approach in their diagnosis and treatment.

- The number of major thrombotic events in PV patients that occurred prior to (30.32%) or during the follow-up (14.83%) was evaluated retrospectively.
- We conducted a separate analysis and evaluation of each and every CV and cerebrovascular event which are usually presented as aggregate data in large-scale trials.
- Regarding cerebrovascular complications in PV, the majority of cerebrovascular complications were chronic ischaemic white matter lesions in our cohort of PV patients. Mild cerebral atrophy was also a frequent finding. The clinical presentation of cerebrovascular events was predominated by lacunar syndromes or VBI. Most of the patients presented at least two serious conventional vascular risk factors, which were supposed to have an impact on both the clinical course of the disease and the morphological alterations seen on brain imaging. These findings suggest that PV predisposes to small vessel cerebral disease manifested primarily as lacunar syndromes, despite the simultaneous presence of additional vascular risk factors in most patients.
- Regarding CV complications, NSTEMI was observed. Most patients had at least two conventional vascular risk factors. Our findings led us to suppose that the early diagnosis followed by percutaneous coronary intervention and an aggressive and personalized management of CV risk factors may be effective in the prevention of subsequent vascular events. The importance of a close co-operation between the haematologist and specialists in the field of vascular medicine is emphasized.
- The contribution of CV risk factors as newly hypothesized additional risk factors to subsequent thrombotic complications in PV was demonstrated in our patient population. Our findings clearly indicate the importance of the identification and consideration of these risk factors in a more accurate and individualized risk-guided thrombosis management in PV.

5.2 SYSTEMIC MASTOCYTOSIS

Analyses in our SM patient group were driven by the orphan nature and the diagnostic, therapeutic, and prognostic difficulties usually seen in this condition. The multidisciplinary significance of SM lies in its challenging diagnostic aspects and characteristic multi-organ nature.

- As part of our research, we analyzed bone marrow histological features, molecular characteristics, and laboratory and clinical parameters at presentation in a large cohort of SM patients.
- The frequency of *KIT D816V* mutation in our study population was evaluated by SM subtype. The established occurrence of this particular mutation and its impact on the clinicohaematological findings was compliant with general literature data.
- Life expectancy of SM patients was compared to age- and sex-matched controls. In addition, the prognostic relevance of the 2008 WHO classification of SM in the investigated patient population was evaluated. Our analyses on Hungarian patients revealed that the survival in SM (including all subtypes) is worse than that expected in the age- and sex-matched Hungarian population. The life expectancy of patients with ISM was excellent whereas SM-AHNMD and ASM groups had a reduced median survival. The distribution of subtypes and the survival pattern seen in our SM study population was similar to that reported in the largest trial previously published.
- Epidemiological data on SM, an orphan Ph-negative MPN are only sparsely available in the literature. With our research, we provided important new data on SM and quantified its cumulative incidence in the South Great Plain region of Hungary.

5.3 HYPEREOSINOPHILIC SYNDROME

HES is a particularly heterogeneous entity with a wide range of clinical manifestations and a typically multi-organ presentation, facilitating a multidisciplinary thinking in its management

- We first performed investigations with 3DSTE, a novel non-invasive cardiac diagnostic tool in patients with HES. All HES patients and their age- and sex-matched healthy controls underwent complete 2D Doppler echocardiography and 3DSTE.
- On 3DSTE, increased LA volumes and LA-SVs were demonstrated in HES patients, accompanied by reduced LA-CS values. These findings suggest a structural and functional LA remodelling in the evaluated patients. The diagnostic work-up requires a close and effective multidisciplinary co-operation between the cardiologist, neurologist, the neuroradiologist, and the haematologist in order to achieve an early and precise diagnosis and a successful management of cases with hypereosinophilic syndrome.

The diagnosis and treatment of all three investigated Ph-negative MPDs is highly complex, calling for a multidisciplinary collaboration among several clinical and non-clinical divisions.

6 REFERENCES

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