Diagnostic workflow for hereditary erythrocytosis and thrombocytosis

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Diagnostic workflow for hereditary erythrocytosis and thrombocytosis

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Abstract

In the patient presenting with an elevated blood count who does not have an acquired clonal disorder causing a myeloproliferative neoplasm, hereditary erythrocytosis or hereditary thrombocytosis needs to be considered as a possible explanation. A young patient and /or those with a family history of myeloproliferative neoplasm should specifically raise this possibility. Among the causes of hereditary erythrocytosis are mutations in the genes in the oxygen sensing pathway and high-affinity hemoglobins. Hereditary thrombocytosis has been shown to be accounted for by mutations in *THPO*, *MPL* and *JAK*2 genes. In those who have a possible hereditary erythrocytosis or thrombocytosis, the investigative pathway includes specific investigation to rule out the more common acquired clonal disorders and if indicated other secondary causes, measurement of specific cytokines as indicated and search for specific identified molecular lesions which have been shown to cause these hereditary disorders. There remain individuals who appear to have an hereditary disorder in whom a genetic lesion cannot currently be identified.

Learning objectives

- Discuss the causes of erythrocytosis and thrombocytosis
- Explain the role of familial factors in myeloproliferative neoplasms
- Appraise the diagnostic pathway to investigate for an hereditary erythrocytosis or thrombocytosis

Clinical case

A 27-year-old Caucasian male was referred to hematology complaining of fatigue and having been found to have an abnormal blood count. He had no previous medical history of note. No family

history of hematological disorder was elicited. He was a non-smoker and consumed 16 units of alcohol per week.

On examination he was overweight but had no abnormal findings. Blood count showed an hemoglobin (Hb) 193g/l, hematocrit (HCT) 0.59 l/l, mean cell volume 89 fl, mean cell Hb 29.9pg, mean cell Hb concentration 333g/l, white blood cells 6.5 × 10^9/l with normal differential count, platelet count 167 × 10^9/l. Further initial investigations were a serum erythropoietin (EPO) 15.9mIU/ml (NR 2.5-10.5), negative for the *JAK2* V617F mutation, red cell mass 146% of predicted and Hb electrophoresis normal.

Introduction

Since, Dameshek introduced the concepts of the myeloproliferative disease in the 1950s definitions of these diseases have been available starting with the definitions of polycythemia vera (PV) and essential thrombocythemia (ET) used in the original Polycythemia Vera Study Group trials. These definitions suggest limits for the Hb and HCT above which there is by implication an erythrocytosis. Similarly limits for platelet counts above the defined limit suggest that there is a thrombocytosis.

An absolute erythrocytosis is present when the red cell mass is greater than 125% of predicted (if this test is available) and it can also assumed that if the HCT is greater than 0.60 in an man and 0.56 in a woman then there is an absolute erythrocytosis[1] but anyone with a Hb/HCT above the limits for the definitions of PV may have an erythrocytosis and needs further investigation. Similarly, for platelets counts in the range of ET further investigation may be indicated.

Some family history of myeloproliferative neoplasm is often revealed by patients and this needs to be assessed in the context of an abnormal blood count. This review will focus on the hereditary causes of myeloproliferative diseases (diseases rather than neoplasms as these are germ-line alterations rather than acquired clonal neoplasms) including erythrocytosis and thrombocytosis and then explore the diagnostic pathways for these disorders.

Familial myeloproliferative neoplasms

In sporadic cases of myeloproliferative neoplasms (MPN) with careful inquiry 7% to 8% have other family members with MPN[2]. At population level a number of germline predisposition alleles have been identified such as the *JAK2* 46/1 haplotype[3]. In the Icelandic population and in an Italian cohort the germline sequence rs2736100 in the *TERT* gene associates with MPN[4,5]. Multiple germline variants, have been described in MPN cohorts where the variants predispose to MPN[6] and recently predisposition alleles were shown to be associated with MPN and age-related clonal hematopoiesis[7].

There have been several extended families described where the germline variant leading to the genetic predisposition is identified. In these cases of familial clustering of MPN the acquired MPN is indistinguishable from sporadic MPN, with a variety of different MPNs and acquired driver mutations in the same family. In 4 families from the same geographical location, a germline copy number variation, leading to germline duplication of *ATG2B* and *GSKIP* predisposes to MPN and progression to myeloid malignancy[8]. The *RBBP6* gene has been shown to be the candidate for genetic predisposition to MPN in one extended family[9].

Diagnostic criteria for Polycythemia vera in 2019

In the 2017 revision the World Health Organisation (WHO) have published revised criteria for the definition of PV where an Hb >165g/l (men) and 160g/l (women) or HCT > 0.49 (men) and 0.48 (women) or other evidence of increased red cell mass, *JAK2* mutation and a bone marrow biopsy showing panmyelosis for are the major criteria for a diagnosis of PV[10]. These definitions give limits which have changed over time. The current definition uses lower levels of Hb and HCT than previous WHO PV criteria and is based on retrospective studies which suggest that these limits can discriminate between PV and ET[11]. It also assumes that it is possible to reliably discriminate between PV and ET on the basis of the morphological appearance of the bone marrow. The British Society for Haematology (BSH) have reviewed the evidence and suggested that criteria for the diagnosis of PV

should be high HCT >0.52% (men), >0.48% (women) or a raised red cell mass (>25% above predicted) and a mutation in *JAK2* with more detailed criteria for the very rare *JAK*2 mutation negative[12]. These criteria are likely to be more practical in defining those with PV without flagging too many of those with Hbs within the normal range as in need of investigation. However what all these criteria identify is those with clonal disease and erythroid proliferation who have the acquired disease, PV. If an erythroid proliferation/erythrocytosis is identified but not an acquired clonal disorder then other causes for the erythrocytosis may need to be investigated.

Causes of an erythrocytosis

An erythrocytosis can be primary where there is an intrinsic disorder in the erythroid progenitor cells or secondary where an external cytokine erythropoietin (EPO) drives increased red cell production. Primary and secondary causes can be further divided into hereditary (or congenital) and acquired.

The main and predominant cause of primary acquired erythrocytosis is PV where usually there is an acquired *JAK2* clone driving red cell production. Primary hereditary causes are very rare with the main one of note being mutations in the *Erythropoietin Receptor* gene (*EpoR*).

There are many acquired secondary causes of erythrocytosis. These can be hypoxia driven where hypoxia leads to an increased EPO and a drive to red cell production. Such hypoxia can be a central process such as chronic lung disease or right-to left cardiopulmonary shunts or local renal hypoxia such as renal artery stenosis. Secondary acquired erythrocytosis can also be due to pathological EPO production. A variety of tumors have been described where the tumor is a source of EPO including cerebellar hemangioblastoma, renal cell carcinoma, hepatocellular carcinoma and uterine leiomyoma. Exogenous administration of EPO and related substances such as androgens can also lead to a secondary acquired erythrocytosis. Many other factors can lead to acquired secondary erythrocytosis such as living at high altitude, obstructive sleep apnoea, smoking and other behaviours. However, secondary hereditary erythrocytosis can be caused by a number of different

defects including mutations in the genes in the oxygen sensing pathway and high oxygen-affinity hemoglobins.

Hereditary erythrocytosis

There are a number of possible hereditary causes of primary or secondary erythrocytosis (Table 1) EPO links to a receptor on the cell surface, the erythropoietin receptor (EpoR). The proteins JAK2 and STAT5 then autophosphorylate, STAT5 dimerises, translocates to the nucleus, and triggers downstream signalling and production of red cells. Then the process is turned off when a further protein SHP-1 attaches and down-modulates the receptor. However, mutations occur in the *EpoR* gene leading to a truncated protein receptor which has lost the SHP-1 docking site. Thus, when EPO attaches to the mutated receptor it is switched on but cannot be switched off and therefore continues to drive red cell production without further EPO stimulation[13]. Therefore in the presence of low EPO levels with a mutated receptor which is switched on results in increased red cell production. At least 11 mutations in the *EpoR* have been described with erythrocytosis[14].

The *SH2B3* gene encodes for the SH2B3 protein, also known as lymphocyte adaptor protein (LNK), which is involved in cell signalling and is a negative regulator of cytokine signalling by attenuating JAK activation. Mutations have been described in *SH2B3* in MPNs. These mutations result in a defective LNK protein which does not act as a negative regulator of the JAK/ STAT pathway downstream of the cytokines and thus lead to increased erythropoiesis, a primary erythrocytosis (with an associated low EPO level). In several cases the mutation was shown to be in the germline and therefore germline *LNK* mutations could be a cause of hereditary erythrocytosis[15].

Mutations of the oxygen sensing pathway

The human organism has a sensitive mechanism for sensing oxygen levels and responding to hypoxia. In conditions of normal oxygen levels, the prolyl hydroxylases (PHD)s hydroxylate hypoxia-inducible factor (HIF) and bind the von-Hippel-Lindau tumour suppressor protein (VHL). Ubiquitination and degradation of HIF then occurs in the proteasome and thus low HIF levels are maintained.

In hypoxia, less hydroxylation occurs, HIF escapes VHL mediated degradation. Levels of HIF then rise, the protein moves to the nucleus and binds to the hypoxia response element in the 3' region of the target genes. This then leads to HIF regulated transcription and production of a number of proteins including EPO. Mutations of the genes in this pathway can therefore lead to failure of HIF breakdown and increased EPO drive. The patients therefore have as elevated or normal EPO levels in the presence of an erythrocytosis.

The first defects in the oxygen sensing pathway were discovered in the *VHL* gene. A homozygous mutation in the *VHL* gene C598T was identified in a large cohort of individuals with erythrocytosis in the remote upper Volga region of Russia, Chuvashia[16]. The mutant protein was shown to have reduced activity as a negative regulator of HIF-1 dependent gene transcription and increased expression of HIF-1 regulated genes target genes including the *Erythropoietin* (*EPO)* gene. The homozygous mutation in the *VHL* gene has been identified in other patients with congenital erythrocytosis from other areas and a few compound heterozygotes of the *VHL* gene with erythrocytosis have been described. Recently new *VHL* exon and complex splicing alterations have been described in some cases of hereditary erythrocytosis[17].

An *EGLN1* (*PHD*2) gene was discovered in a family with erythrocytosis, a heterozygous change C950G leading to a protein alteration of proline to arginine at codon 317. Affected siblings had erythrocytosis with normal or increased EPO levels[18]. *In vitro* studies showed that the mutation had abnormal activity which would cause erythrocytosis and a mouse model provided further evidence of the mutation as a cause of erythrocytosis[19]. A number of further mutations in *PHD2* have been documented in individuals with erythrocytosis[14]. In one case an individual, thirteen years after presentation, was found to have a paraganglioma[20]. The mutation was also found in the tumor tissue and absence of the wild type *EGLN1* allele, thus loss of heterozygosity. This suggests in this case that *EGLN1* was acting as a tumor suppressor gene.

An *EPAS1* (*HIF2A)* gain-of –function mutation which was identified in 3 generations of a family associated with erythrocytosis, a G1609T change leading to a change at codon 537 from glycine to tryptophan. *In vitro* studies showed that the altered protein bound PHD2 and VHL differently than the wild type protein, degraded it more slowly, and induced downstream genes[21]. Other mutations in *EPAS1* associated with congenital erythrocytosis have been identified in other kindred.

Thus, a number of genes in the oxygen sensing pathways have been shown to be mutated and cause hereditary erythrocytosis[14].

The clinical presentation of these cases is not clear. There are some features described such as early development of varicose veins in Chuvash polycythemia. Early severe thrombotic events and emerging pulmonary hypertension are noted with other defects. Such clinical signs may alert to the possibility of an oxygen sensing pathway mutation but there is no clear phenotype.

Recently a gain-of-function mutation in the *EPO* gene itself has been described leading to a familial disorder with high EPO levels[22].

High oxygen-affinity haemoglobins

Oxygen is transported to the tissues bound to hemoglobin. The oxygenation and deoxygenation of hemoglobin occurs at the heme iron binding site and the affinity for oxygen depends on the nature of the hemoglobin. The hemoglobin oxygen dissociation curve describes this relationship. An high oxygen-affinity hemoglobin, has a left shifted oxygen dissociation curve as oxygen is tightly bound. At tissue level this results in relative hypoxia, EPO production and secondary erythrocytosis. Approximately 100 high oxygen-affinity variants have been described with both α and β globin gene mutations resulting in stable and unstable hemoglobins. These have an autosomal dominant inheritance[23].

One per cent of hemoglobin is normally in the methemoglobin form which impairs oxygen binding and transport. Presence of a large amount of methemoglobin leads to cyanosis and a compensatory erythrocytosis develops. Congenital methemoglobinaemia can arise either because of a deficiency

cytochrome b_5 reductase or an abnormal M hemoglobin. NADH-cytochrome $b_5$reductase catalyses electron transfer from NADH to cytochrome b_5 and is encoded by the *CYB5R3* gene. Over 40 mutations of this gene have been described and inheritance is autosomal recessive. Type 1 mutations lead to a defect in the erythrocytes only whereas type 11 mutations have accompanying neurological defects[24].

Other secondary causes of hereditary erythrocytosis

Binding of 2,3 bisphosphoglycerate (2,3-BPG) to hemoglobin converts the hemoglobin molecule to a low oxygen-affinity state shifting the oxygen affinity curve to the right. Therefore, deficiency of 2,3-BPG moves the oxygen dissociation curve to the left, less oxygen is delivered to tissues and a compensatory erythrocytosis results. In the glycolytic pathway, the production of 2,3-BPG involves the conversion of 1,3 BPG to 2,3 BPG catalysed by bisphosphoglycerate mutase (BPGM). Mutations in the *BPGM* gene lead to an abnormal functioning BPGM and deficiency of 2,3-BPG[25].

Families have been identified with mutations in the *SLC30A10* gene who have the syndrome of hepatic cirrhosis dystonia, erythrocytosis and hypermanganesemia. Manganese induces *EPO* gene expression and increased EPO levels are seen in these patients causing the erythrocytosis[26]. Erythrocytosis has been also reported in families who have been described with increased ATP levels associated with low 2,3-BPG levels with autosomal dominant inheritance[27].

Diagnostic criteria for essential thrombocythemia

The WHO has recently revised the criteria for ET. The major criteria are, the platelet count $\geq 450 \times 10^9$/l, proliferative bone marrow appearances with megakaryocyte predominance, not meeting the criteria for other myeloid neoplasms and presence of a *JAK2*, *CALR*, or *MPL* mutation, and the minor criteria presence of a clonal marker or no evidence of a reactive thrombocytosis. All 4 major or 3 major and 1 minor criteria are required to make the diagnosis. This is again showing evidence of an acquired clonal neoplasm. The lower limit of the platelet count is unchanged from the previous WHO

criteria and in patients with a raised platelet count above 450 × 10^9/l who do not fulfil the may have other than an acquired clonal neoplasm[10].

Causes of thrombocytosis

A platelet count above the normal range can be caused by a primary disorder arising in the bone marrow compartment. It can also be secondary or reactive and the result can be spurious where other cellular factors such as microspherocytes, schistocytes or bacteria are being mistakenly counted as platelets. Numerous reactive secondary causes can lead to an elevated platelet count including infection and inflammation, post-operative and following tissue damage, and hyposplenism. The platelet count is often elevated with hemorrhage, iron deficiency, malignancy, hemolysis and can rebound following myelosuppressive therapy and the presence of any such condition needs to be considered in the patients presenting with an elevated platelet count.

Primary acquired thrombocytosis in seen with the acquired clonal disorder essential thrombocythemia but it is also seen in other myeloid malignancies including PV, primary myelofibrosis and prefibrotic myelofibrosis, myelodysplasia with isolated del5q and myelodysplastic (MDS)/MPN with ring sideroblasts and thrombocytosis, chronic myeloid leukemia and MDS/MPN unclassified. There are however, rare individuals with a primary thrombocytosis who may have a hereditary or congenital, germline cause for the thrombocytosis.

Hereditary thrombocytosis

Rare families have been described with a clearly inherited thrombocytosis (Table 2). The *thrombopoietin* (*THPO*) gene was isolated in 1994 and following this a number of alterations in the *THPO* gene were discovered in families with autosomal dominant hereditary thrombocytosis. These gene alterations result in translational inhibition of *THPO* mRNA and elevated thrombopoietin (TPO) levels in serum resulting in thrombocytosis. Affected family members in these kindreds had elevated TPO levels[28]. Associated distal limb defects are described with some of these mutations indicating a role for TPO in vasculogenesis[29].

The *myeloproliferative leukaemia* virus oncogene (*MPL*) codes for the thrombopoietin receptor MPL. A polymorphism in *MPL*, *MPL* Baltimore was found to be associated with mild thrombocytosis in African-American women who were heterozygous for the polymorphism with extreme thrombocytosis in homozygotes. This polymorphism is restricted to individuals of African-American decent[30]. Families have been described with a point mutation in the transmembrane domain of the *MPL* gene with an autosomal dominant inheritance. This mutation activates both intracellular signalling and cell survival. This has been found in a number of Italian children with hereditary thrombocytosis and follow up of this cohort demonstrated a large number of major thrombotic events and with aging development of splenomegaly and bone marrow fibrosis[31]. In Arab families a point mutation in *MPL* (P106L) is causative for hereditary thrombocytosis with high TPO and platelets levels in homozygotes and mild thrombocytosis in heterozygotes[32]. Another, nearby point mutation (R102P) is also described associated with thrombocytosis in heterozygotes (homozygotes have congenital amegakaryocytic thrombocytopenia). It is thought in the heterozygotes subnormal cell surface expression of wild type MPL in platelets induces defective TPO clearance[33].

The *Janus Kinase 2* (*JAK2*) acquired point mutation V617F is associated with PV and ET and other acquired mutations in *JAK2* exon 12 are also seen in PV. However, in recent years other mutations germline mutations in *JAK2* have been associated with hereditary thrombocytosis (Table 2) with autosomal dominant inheritance. *JAK2*V617I was found in a family with high penetrance hereditary thrombocytosis. The mutant is shown to induce cytokine hyperresponsiveness which would produce the phenotype[34]. At *JAK*2 residues other than 617 mutations have been described, R564Q in a family[35] and also H608N[36]. In other families, further germline *JAK*2 mutations have been found and demonstrated to be active in altering signalling R867Q and in one family 2 *JAK*2 mutations located in cis both in the pseudokinase and kinase domains. These mutants have altered constitutive signalling, leading to growth factor independence and hypersensitivity to TPO[37].

Of note, a patient with 2 germline mutations E846D and R1063H one inherited from each parent is described with erythrocytosis and atypical megakaryocytes but a normal platelet count suggesting that various germline *JAK*2 mutations have roles in MPNs[38].

Pathway for investigation

The patient referred with an elevated blood count may have an obvious diagnosis of sporadic MPN with an identified driver mutation. However, familial MPN, reactive and secondary causes and then hereditary reasons need to be considered. In the patient referred, the first necessity is to take a careful history from the patient with particular care to enquire about family history of likely myeloproliferative neoplasm and vascular disease. Other factors which may suggest reasons for the elevated blood count such as drug use including recreational and lifestyle factors should be considered. A confirmatory blood count after a suitable interval from the original one is required to confirm a sustained elevated count.

Initial investigations would then include a screen for mutations in *JAK*2 and proceeding to *CALR* and *MPL* if appropriate. A serum EPO will show either a low or a raised or inappropriately normal suggesting either a primary or secondary erythrocytosis. A bone marrow examination with cytogenetic examination should be considered to see if any MPN is present and to confirm any such diagnosis. This should be undertaken in any patient in any patient in whom the explanation for the abnormal blood count is not obvious. Other initial investigations could include iron studies to consider reactive causes such as iron deficiency and C reactive protein (CRP) as a marker for inflammation (Table3: Stage 1 investigations)

Having carried out stage 1 investigations, and considered the possibility of a hereditary erythrocytosis or thrombocytosis further investigation is directed at identifying the hereditary cause and ruling out any alternative explanation if appropriate. With an erythrocytosis a red cell mass is a useful way of confirming the presence of the erythrocytosis although this is becoming an increasingly difficult test to obtain. Imaging may be indicated to search for lesions which would

include renal ultrasound, CT scan of abdomen and neuroimaging. A venous P_{50} measurement will show if the oxygen dissociation curve is shifted and hemoglobin electrophoresis if an abnormal hemoglobin. Specific sequencing of genes of the oxygen sensing pathway for known mutations can be carried out. However, whole genome sequencing[39] if available will find mutations and next generation sequencing (NGS) panels for testing genes which can cause hereditary erythrocytosis including the hemoglobin genes are becoming available and it is likely that such NGS panels will replace some of the other tests.

With a possible hereditary thrombocytosis, having eliminated possible reactive causes, specific tests could include a TPO level as this is elevated with some of the hereditary causes and if found is useful confirmation. Full sequencing of *THPO*, *MPL* and *JAK*2 is required to search for the described defects to confirm an hereditary cause (Table 3: Stage 2 investigations).

Investigation for familial myeloproliferative neoplasm

Careful history taking in individuals with sporadic MPN suggests that a number will have a family history of MPN as with at least 2 cases of MPN in a family tree. It would not be routine practice to investigate the genome to identify the genetic predisposition and this would currently only be done as research investigations. However, identification of such family cases may be useful as the suggestion is that a blood count should be performed in health relatives to identify early an MPN.

Idiopathic erythrocytosis

At the end of investigations, there remain patients who have an erythrocytosis in whom no cause can be identified. These patients are termed idiopathic erythrocytosis. The number of such patients has diminished with time as new reasons for an erythrocytosis are discovered and new investigations such as NGS become available, they should be explored in those labelled idiopathic erythrocytosis. These patients need to be followed up long term so that they can be investigated further and long term outcomes established.

Return to the clinical case

Initial investigation of our patient demonstrated a clear erythrocytosis with a raised EPO level suggesting a secondary erythrocytosis. Further testing did not reveal any mutations in the oxygen sensing genes which were investigated as mutations in individual genes were described. A P_{50} of 23.91mmHg (normal 27-33) was found suggesting a left shifted oxygen dissociation curve. However, as part of a whole genome sequencing research project, DNA sequence analysis detected a heterozygous G>A substitution at nucleotide c.269 in the *BPGM* gene in the patient. This change was also detected in his mother but not his father. An assay for 2,3 BPG, showed lower levels in the patient and his mother compared to controls confirming the functional effect of the mutation. The mother did not have an elevated Hb but at an Hb of 155g/l her Hb was at the upper end of the normal range for a female[40]. This patient with erythrocytosis was found to have a causative inherited mutation in the *BPGM* gene.

Conclusion

A young patient and /or a possible family history of myeloproliferative neoplasm can suggest a possible hereditary cause for a raised blood count. This should be investigated by specific genetic investigations after other initial investigations to rule out the more common acquired disorders. On completion of investigations, there remain individuals in who a hereditary disorder is likely but in whom no abnormality can be identified. These individuals are subjects for future research investigation.

Conflicts of interest: Italopharma: advisory committee. Novartis: Honoraria and speakers bureau. Daiko Sanyo: advisory committee.

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Table 1: Causes of hereditary erythrocytosis

Primary

EPO receptor mutations

SH2B3 (LNK) mutations

Secondary

Oxygen sensing pathway defects

-*EGLN1* (*PHD2)*

-*VHL*

- *EPAS1 (HIF2A)*

Gain-of-function mutation *EPO* gene

High oxygen-affinity hemoglobins

Methemoglobinaemia

Bisphosphoglycerate mutase deficiency

SLC30A10 mutations with hypermanganesemia

Hereditary ATP increase

Table 2: **Causes of hereditary thrombocytosis**.

THPO- Thrombopoietin, *MPL*-TPO receptor, *JAK2- Janus kinase 2*.

Gene	Mutation	Reference
THPO		ENST00000647395.1
	c.-47delG	
	c.13+2T>C	
	c.141+1G>C	
	c.-31G>T	
MPL		ENST00000372470.8
	p.(Lys39Asn)	
	p.(Arg102Pro)	
	p.(Pro106Leu)	
	p.(Ser505Asn)	
JAK2		ENST00000381652.3
	p.(Arg564Gln)	
	p.(His608Asn)	
	p.(Val617Iso)	
	p.(Ser755Arg)/p.(Arg928Gln)	
	p.(Arg867Gln)	

Table 3: Investigations

Stage 1

Detailed history including family history

Repeat confirmatory Full blood count

Serum EPO

*JAK*2, *CALR, MPL* mutation screen

Bone marrow aspirate and trephine

With Cytogenetics

CRP

Iron studies

Stage 2

Erythrocytosis	Thrombocytosis
Red cell Mass	Serum TPO
Imaging	Sequencing of *THPO, MPL* and *JAK*2 genes
Overnight oximetry	
P_{50} measurement	
Hb electrophoresis	
Sequencing for mutations in genes Oxygen sensing pathway	
Next generation sequencing panel for hereditary erythrocytosis	