Review & Hypothesis: Hematologic Oncology
J Biophys Hematol Oncol **1**, 6:1-164 (2010)

Anemia, Polycythemia and Leukemia (1): molecular biology and functional interrelationships of hematologic disorders

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Abstract

In the following communication, we emphasize the interconnectedness of hematologic neoplasms and their developmental processes, as well as propose new diagnostic classifications for a variety of hematologic disorders. We sought to produce a comprehensive and accurate review of hematologic disorders based on updated medical research, but also connect them as transitional forms of distinct neoplastic processes ("subvectors") that, despite the various lineage-specific phenotypes, repeat a fundamental auto-oncogenic progression (an auto-oncogenic vector) of pre-neoplastic, chronic, subacute and acute (or terminal) stages. Auto-oncogenic theory suggests that the main causation of acquired post-adaptive malignancies involves hypoxic or hypoxia-like factors that chronically impair respiratory metabolism and adversely affect, first and foremost, the red blood cell compartment, both in its functional role and in its formative process (erythropoiesis) [1]. In the present communication, we extend the proposed etiology to hematologic neoplasms, to include deregulated humoral and cellular auto-immune responses to RBCs and their precursors as one of the main pathways, if not the dominant one, for the induction of myeloid and lymphoid neoplasms.

First, we review the multiplicity of disorders that result in anemia or in erythroid/myeloid polycythemias, with an emphasis on the problems associated with determining the correct etiology of *Polycythemia vera* (PV) and the so-called myelodysplastic syndrome (MDS) that includes refractory anemias. We suggest that the chronic myeloproliferative disorders (PV; Essential Thrombocytosis, ET; Primary Myelofibrosis, PMF; and other neoplasms, including Refractory Anemia with Ring Sideroblastosis and Thrombocytosis, RARS-T) should be viewed as auto-oncogenic, chronic blood neoplasms, that differ in their phenotype by altered lineage-specific cytokine responses, but are simply variants of a myeloid stem cell neoplasia that has in common the *JAK2V617F* adaptive mutation.

In the second part, we review the myeloid leukemias, and the lymphoid and lymphomatoid neoplasms, but, with a few exceptions, restrict ourselves to auto-oncogenic malignancies - unlike our approach to the anemias and the polycythemias, which included all possible etiologies, not just auto-oncogenic ones.

 ISSN 1920-3799

In the domain of the myeloid leukemias, we stress in particular:

(1) the existence of an erythroleukemic vector that encompasses chronic phases like PV or Refractory Anemia with Ring Sideroblastosis (or, still, the early stages of typical and atypical CML, which often present a chronic erythroleukemic picture), pre-acute crises like those observed in PMF and MDS, and acute phases like those of acute erythroblastic leukemia and other forms of acute myelogenous leukemia; and

(2) the progression of this vector to encompass a wider myeloid leukemic vector, joining in with the auto-oncogenic, Philadelphia chromosome-positive vector linking chronic myeloid leukemias, acute myelogenous leukemias, B cell acute lymphoblastic leukemia and biphenotypic acute leukemia.

In the domain of the lymphoid leukemias, we stress their connection, together with the hemolytic anemias caused by cellular or humoral auto-reactivity, to disturbed auto-immunity whose dominant target is the erythroid compartment. We suggest that this anti-RBC reactivity is a neoplastic response to the biological degradation of RBC function and formation. If initiation of disturbed poietic or immunologic auto-oncogenic responses is promoted by hypoxia-induced RBC fragility and dysfunctionality, then it becomes clear why altered erythropoiesis and RBC clearance dominant in the etiology of acquired blood neoplasms.

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COMMUNICATION

"Despite these advances in knowledge, patients still develop immune haemolytic anaemias, and their treatment may present as an urgent and serious problem. What advances in knowledge of benefit to patients can be expected in the future? The essential problems remain to be fully solved. Why and how does self-tolerance to one's own erythrocyte antigens break down and what can be done to contain the breakdown?"

Prof. Emeritus J. Dacie, 2001

"Attempts to define the natural history of polycythemia vera then [80 years ago when it was discovered] as now have been frustrated not only by the low incidence of the disorder but also its chronic nature which precludes most physicians from seeing more than a few of these patients or even following them for a sufficient duration to encounter the full scope of the disease"

J. Spivak, 2002

"[...] If we examine these various [myeloproliferative] syndromes [...] originating from bone marrow cells, as a group, we find it difficult to draw any clear-cut dividing lines; in fact, so many 'transition forms' exist that one may with equal reasonableness call a single condition by at least two different names. (...) Perhaps it is possible to resolve all of these dilemmas, conflicts, antagonisms and confusions by considering, not that the various conditions listed are different, but that they are closely interrelated."

W. Dameshek, 1951

"You canker-blossom, you thief of love!"

W. Shakespeare, "A Midsummer Night's Dream"

"Ce que nous avions compris, nous ne sommes pas allés le dire à la télévision. Nous n'avons pas aspiré aux subsides de la recherche scientifique, ni aux éloges des intellectuels de journaux. Nous avons porté de l'huile là où était le feu."

G. Debord, 1978

INTRODUCTION :
FUNDAMENTALS OF HEMATOPOIESIS AND THE BLOOD SYSTEM

1. The blood system and blood cells

The present and follow-up essay is intended for hematologists, oncologists and informed readers. Accordingly, we thought best to provide a simple introduction for the latter regarding the blood system and its functions.

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The blood system encompasses at once the energy capture and distribution system of vertebrates, and their defence system against infection and toxemia. Blood flow supplies oxygen, essential nutrients and vitamins, hormones or cytokines (growth and differentiation factors), essential ions and other key biological molecules to tissues throughout the body. Blood also supplies both cells and antibodies involved in defending the body from infection, and in removing toxins and waste metabolites. Whole blood is made up of four components - the red blood cells (called RBCs or erythrocytes), the white blood cells (called WBCs or leukocytes, from *leukos*, Gr. for white), the clotting "cells" (called platelets), and a clear fluid called serum in which all the cells circulate.

All the cells of the blood system develop from single, multipotential stem cells through a lineage-producing process called hemopoiesis, which involves both controlled proliferation and cumulative impulses of specific and gradual differentiation along distinct lineages. The entire process is schematized in **Fig. 1**. The main lineages are myeloid and lymphoid, with the myeloid lineage comprising the monocytic, granulocytic, erythrocytic and megakaryocytic (platelet-producing) sublineages. The mature, differentiated blood cells characteristic of each lineage and sublineage are shown at the bottom of **Fig. 1**.

The RBCs are the essential energy conveying element of the blood system. They do this by employing a special iron-rich pigment protein with catalytic properties called hemoglobin (Hb). Mature RBCs are packed with hemoglobin. Human RBCs are so packed that they have no nucleus (which is extruded during RBC maturation), have no DNA or DNA/RNA machinery, and no mitochondria (intracellular organelles responsible for aerobic respiration). Hemoglobin noncovalently picks up oxygen (becomes oxyhemoglobin) as the blood passes through the lungs, transports it throughout the body, and releases it to organs and tissues (becomes deoxyhemoglobin). Oxygenated blood flows through the arterial network. In the return path - the venous network of vessels - hemoglobin picks carbon dioxide to be released in the lungs, during exhalation. Whatever the cause (including hemolysis or excessive RBC destruction), anemias are characterized by a lack of properly matured RBCs (thus note that anemia may coexist with a normal number of RBCs, just not a normal number of "properly matured" RBCs). An excess of RBCs, whatever the cause, is technically called a polycythemia, though other terms are often employed, such as erythrocytemia, erythremia or erythrocytosis.

Platelets or clotting "cells" are not real cells but very small (2 to 4 μm) enucleate cytoplasmic fragments (or cytoplasts) from very large cells in bone marrow - the platelet progenitors called megakaryocytes. Platelets are released from marrow when megakaryocytes shed them to the bloodstream. The main function of platelets is to stop bleeding by forming clots. A deficiency of platelets can cause bleeding into any organ or tissues. Unexplained or excessive skin bruises can be a result of

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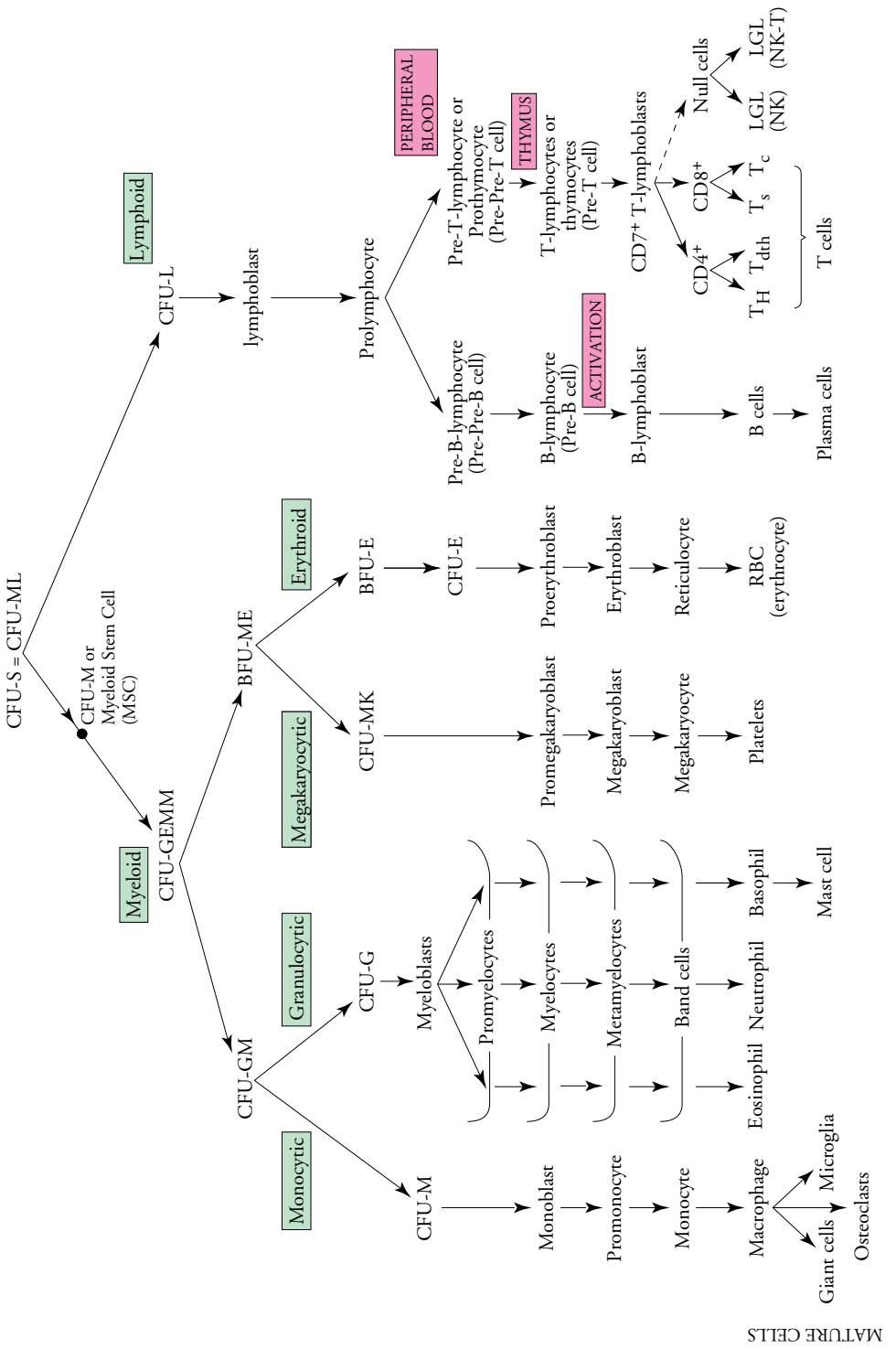

Fig. 1 - Diagram of the stages of hemopoiesis, from the most primitive stem cell, through precursor and progenitor cells, to end-product mature blood cells.

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platelet shortage.

WBCs or leukocytes are in charge of defending the body against disease-producing viruses, bacteria and fungi, as well as mopping up toxins and poisons. Leukocytes fall into two main groups or lineages - myelocytes and lymphocytes.

Myelocytes in turn subdivide into two major groups, the monocytes and the granulocytes. The latter constitute 60-70% of all WBCs, with 90% of all granulocytes being neutrophils. Upon activation, monocytes and granulocytes transform into phagocytes that combat infection, inflammation and necrosis by engulfing foreign or damaged cells, micro-organisms, viruses and toxic substances in special saccules (lysosomes) that invaginate from their cellular membrane. Activated monocytes are called macrophages; they become amoeboid in shape and motion, crawling through the tissues and interstitial spaces between cells, to destroy foreign particles, while retaining the latter's antigens for presentation to lymphocytes.

Lymphocytes (ca 20% of WBCs) circulate both in the blood stream and in a subsidiary network of the blood system, the lymph system that has separate vessels called lymphatics. Lymphocytes are also found in the lymphoid organs which include the spleen, thymus, thyroid and lymph glands. The lymph system functions as a tissue filtration and drainage system that empties to the bloodstream. A clear fluid, lymph, circulates through a network of nodes and vessels, picks up waste material, and delivers it to the bloodstream for removal from the body.

There are also two main subtypes of lymphocytes (see **Fig. 1**), the T and the B cells, which work in concert with each other and in particular with macrophages, neutrophils and other killer cells. T cells attack foreign cells, virus-infected cells and cancer cells. T cells constitute ca 85-95% of all lymphocytes. The two main subtypes of T cells are the antibody-dependent cytotoxic effector T cells (T_C, referred to in the past as K cells and sometimes as ADCC) that express the CD8 co-receptor, and the helper T cells (T_H) that express the CD4 co-receptor. B cells produce and secrete antibodies, specialized proteins that recognize and bind antigens (biomolecules released by, or present on, infectious micro-organisms or viruses). A shortage of any type of normally-functioning white blood cells can cause an increase in infections and result in disease.

There is a third subtype of lymphocytes (see **Fig. 1**), originally referred to as 'null cells' because it was thought it did not carry either T or B cell markers. As it turned out, this third population of CD56+ [2] large granular lymphocytes (LGL) that can kill nonself cells without prior immunization is heterogenous - comprising both CD3- natural killer (NK) cells and CD3+ cytokine-induced killer cells of the T (sub)lineage known as NK-T cells [3-4]. NK-T cells may express CD4, CD7 and CD8 [5-6], these T cell markers being absent from the NK subset. In turn, the NK subset comprises both CD16+/CD94- and CD16-/CD94+ subpopulations, with the latter being a minority but the domi-

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nant NK cell subset in inflammatory responses and lymph nodes [7-8], where they are activated by T cell-derived Il-2 [9]. NK-T cells are considered to be a subset of the cytotoxic T cells that, like NK cells, express the NK receptors (NKRs: CD158a, CD158b and CD158e, aka killer immunoglobulin-like receptors, KIRs) [10]. Whereas the NK activities of NK and NK-T cells are mediated by the KIRs and CD94, the K activities of cytotoxic T cells are mediated by the Fc receptors.

In general, normal 'null cells' are today considered to comprise NK LGLs, NK-T LGLs, and a minority of blast cells that are either myeloid stem cells or immature pre-pre-T or pre-pre-B cells.

2. The growth of blood cells or hematopoiesis

The clonal growth and differentiation of all blood cells from multipotent bone marrow stem cells is called hematopoiesis. It generates two major cell lineages (see **Fig. 1**): (1) the myeloid line which gives rise to monocytes and granulocytes, as well as to RBCs and megakaryocytes/platelets; and (2) the lymphoid line which gives rise to lymphocytes. Thus, each of the two lineages produces two or more sublineages. For ease of reference, it is these sublineages that are most often referred to as hematopoietic lineages - thus one speaks of "the erythroid lineage", "the T lineage", etc.

In the adult body, RBCs, WBCs and platelets are produced in the bone marrow, and released to the bloodstream after terminal differentiation or maturation. B cells develop in marrow, circulate in blood and mature in primary lymphoid organs (marrow included), becoming activated to engage in clonal expansion upon exposure to a specific antigen. Prior to maturation, the B cell is referred to as a B-cell blast, and its cytoplasm presents both mitochondria and characteristically extensive organellar machinery (SER, RER and the Golgi apparatus) for immunoglobulin synthesis. Many B-cell blasts mature into long-lived plasma cells found in connective tissue and the germinal centres of lymph nodes and spleen, and representing 0.1% of the lymphocytes in circulation. These germinal centre B cells known as "follicle centre cells" are frequently found to be malignantly transformed and tumorigenic in lymphoproliferative disorders [11]. T cells mature in the thymus and lymph nodes. T-cell blasts differentiate into T cells in response to antigen, by separating into granular and agranular groups. The granular T cells (T-cytotoxic and T-suppressor cells) contain prominent mitochondria and the electron-dense bodies that are absent from the agranular T cells (T-helper cells).

In the adult, hematopoiesis begins with the production of very immature pluripotential blood cells (here, "pluripotential" means that these cells retain intact the genetic capacity to express all the differentiated characteristics - the phenotypes - of the distinct cells which make up the hematopoietic lineages), from marrow stem cells that are set into cell cycling. Stem cells are primitive, multipotential or nearly-totipotential cells present in all systems, tissues or organs, which are responsible for the cellular regeneration or replenishment of those systems, tissues or organs. In the hematopoietic system, the stem cell thought to have the greatest potentiality is an hypothetical CFU-ML or

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CFU-S (also referred to a primitive hematopoietic stem cell, PHSC, or just PSC) capable of generating all the myeloid and lymphoid lineages. It is unclear whether such a primitive stem cell also gives rise to the metabolic, secretory and support cells that form the stroma of bone marrow. Some researchers (M. Ogawa [12], A. Axelrad, etc) have suggested that the term CFU-S should designate solely the most primitive myeloid stem cell capable of self-renewal, and typically more primitive than the CFU-GEMM or the CFU-L (see **Fig. 1**). At one time (in the late 1980's and during most of the 1990's), it was thought that this was a CD34+ cell. But in the mouse, the most primitive hematopoietic stem cell is CD34- CD38+ [13]. In human marrow, the PHSCs appear to be TdT-, CD34-, HLA-DR+ [14], but the markers for such primitive stem cells are still undetermined and, in effect, disputed.

Once recruited (set into cycling), marrow stem cells begin to divide (proliferate) to produce pluripotential blood cells having the appearance of blasts (see **Fig. 2**). In turn, these blast cells continue proliferating to generate the "blood progenitor cells" that progressively differentiate along one or more blood lines to produce mature blood cells. In a tightly choreographed dance, the body regulates production and removal of mature blood cells - with the spleen, liver and thymus being in charge of removing dysfunctional and senescent blood cells. Self-regulation of erythrocyte production is a poignant example of this dynamic process: while some RBCs (mean 100-120 days life span) mature in the marrow and are released into the bloodstream to replace those which have become old and worn out, deformed or inefficient (dysfunctional) RBCs are attacked by macrophages and granulocytes in marrow, spleen and liver, and their molecular components digested by these cells and recycled back to the marrow.

There are other normal sites of hematopoiesis in the adult, such as spleen and lymph nodes, but these are normally minor. However, during embryonic and fetal development, blood cells are formed in very different organs, first in the umbilical cord, then the liver, spleen and finally the marrow of long bones.

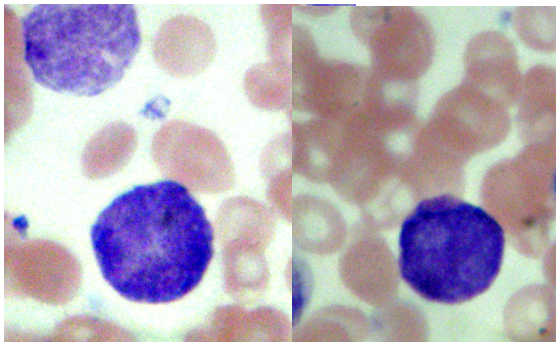

Fig. 2 - Examples of normal primitive stem cells in a blood smear of a bone marrow biopsy. Wright's stain, Nikon Coolpix P5000, 1,000x print mag (left), 875x print mag (right).

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In healthy adults, blast cells are not present in the circulating blood and lymph systems, and in marrow only a small amount of them (<5% of total number of marrow cells) is found at any time. In certain leukemias, this number increases, acute leukemias being characterized precisely by an uncontrolled, excessive proliferation of blasts in marrow, that eventually spills over to the blood-stream.

ANEMIA, POLYCYTHEMIA AND LEUKEMIA

1. The anemias

The concentration or volumetric density (weight/volume) of both hemoglobin and erythrocytes in peripheral blood varies within a typical range. Anemia is technically defined as a condition that encompasses all possible reductions of blood concentration of hemoglobin below the typical range. The deficient concentration of hemoglobin may, in turn, be due to either a lack of erythrocytes or a lack of hemoglobin itself. The diagnostic trigger-signal of anemia is hemoglobin ≤ 11 g/dL.

Technically, as a clinical condition, anemia constitutes a complex and dynamic symptom of a wider disorder rather than a disease per se. In turn, anemia can cause weakness, dizziness, shortness of breath, headaches, and irritability. The group of the anemias includes diseases that present either decreased numbers of erythrocytes, decreased erythrocytic hemoglobin content, or dysfunctional hemoglobin - due to either deficient erythropoiesis (which may be genetic, acquired, infectious or nutritional), excessive hemolysis or a combination of the two. In iron-deficiency anemias or in the anemias of chronic disease, iron concentrations in serum are depressed, whereas in hemolytic disease and iron-overload syndromes they are elevated. The differential for low iron concentrations in serum is the increased Fe-binding capacity of transferrin in iron-deficiency vs its reduced Fe-binding capacity in anemia of chronic disease. Particular forms of anemia are of special interest to the present essay because they can be - and have been at times - analyzed as constituting specific forms of erythroleukemia. This is the case with: the normochromic anemia of early-phase myelofibrosis (MDS, or myelodysplasia) which has traits of a chronic erythrocytosis; the leukoerythroblastic leukemia of late phase MDS, which has traits of an acute erythroblastosis; and the refractory anemias with ring sideroblastosis (RARS), which can be conceptualized as a form of erythroid blast crisis.

1.1. Anemia due to deficient erythropoiesis

Anemias caused by deficient erythropoiesis are easily spotted upon microscopic examination of peripheral blood (PB) RBCs. Besides the characteristic doughnut shape, normal human RBCs have definite ranges of size (volume and diameter) and hemoglobin concentration. In properly prepared,

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fixed and stained spread-film slides of peripheral blood for microscopic examination, RBCs appear to be very uniform in size and color, with very low frequencies of altered shape or reticulocyte (immature RBC) occurrences (1 in 100 to 1 in 1,000). Whereas anisocytosis (heterogeneity of size) is common in various anemias, other anemias present relatively uniform changes in RBC size. If the erythrocytes are smaller than normal, they are are referred to as microcytes (mean cell volume, MCV <80fL/RBC), and if they are larger, as macrocytes (MCV >95fL/RBC). Normal size RBCs are referred to as normocytic or normocytes.

Microcytes are typically the outcome of a disturbed iron metabolism in microcytic anemias, and they maybe hypochromic when their hemoglobin density is low (mean cell hemoglobin, MCH <27pg/RBC). Macrocytes that are not megaloblastic (ie like giant blast cells) are associated with a dysfunctional RBC regulation of plasma-membrane production, whereas megaloblastoid oval-macrocytes are associated with forms of pernicious anemia caused by vitamin (B_{12}, folate and C) deficiencies. Normocytic (also called normochromic-normocytic) anemias are typically, but not exclusively, caused by hypoproliferation and hypoplasia of RBCs; aside from impaired EPO production in renal disease, normocytic anemias are due either to hypometabolic responses to growth factors controlling erythropoiesis, or to generalized marrow disease that evolves from hypoplasia to pancytopenia (aplastic anemia and refractory anemias). Altered erythroid growth factor (cytokine) responses involve EPO signaling, but just as well signaling by other regulatory cytokines such as Insulin-like Growth Factor I (IGF-I), retinoids (vitamin A derivatives), Interleukin-3 (IL-3), Stem Cell Factor (SCF), and still others (see below). To this day, the physiological responses to these cytokines are still poorly understood, largely because of a combination of two factors: the continued practice of utilizing serum in tissue culture, for serum is contaminated with undefined quantities of various cytokines and inhibitory factors that will mask and skew any cytokine investigation; and the frequent use of established cell lines rather than primary cell populations in the investigation of biological responses. As has been repeatedly shown by many medical researchers, altered growth factor responses are best studied in chemically defined serum-free media.

1.1.1. Microcytic Anemias: iron deficiencies, hemoglobinopathies and chronic disease

Total iron body stores range from 2.5 to 3.5 g, with over 2 g being associated with hemoglobin (and ca. 200 mg with myoglobin, and 150 mg with other heme and nonheme enzymes). Disturbed iron metabolism may have a variety of causes. Substantial bleeding - occult or not, including menstrual loss - is sufficient to depress blood hemoglobin content and trigger the production of RBCs with deficient heme or globin synthesis. But blood iron may be simply decreased by inadequate nutritional intake of iron, by an increased dynamic requirement for iron, or a diminished absorption

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of iron, these distinct factors being often combined. Indeed, an increased requirement for iron may well coexist with a decreased intake which, in addition, may be such as to not promote iron absorption. This is underlined by some basic facts pertaining to iron absorption, which is a regulated composite of distinct processes. Iron is best absorbed when it comes already complexed with heme in the food (eg meat) intake. *Most nutritional intake of iron is not complexed with heme*. Dietary nonheme iron is typically reduced to the ferrous state, and subject to interactions with various foods that decrease its absorption. When iron stores are not replete, the concentration of macrophage-released serum ferritin (an iron transporter protein) in blood is inversely related to absorption of nonheme iron [15]. *The only nutritional factor known to increase absorption of nonheme ferrous iron is ascorbic acid* (vitamin C). Nonheme iron must be transferred to mitochondria via a complex formed with transferrin (another iron transporter protein), and the mitochondria insert it into protoporphyrin in order to synthesize heme. As discussed already [1], heme, ferrous iron and vitamin C are all necessary for the respiratory metabolism of tissues, thus mitochondria are their ultimate destination. These particular molecules are also essential for the proliferation and differentiation of erythroid progenitors (BFU-E and above all CFU-E, see **Fig.s 1 & 3**), and for their maturation into RBCs.

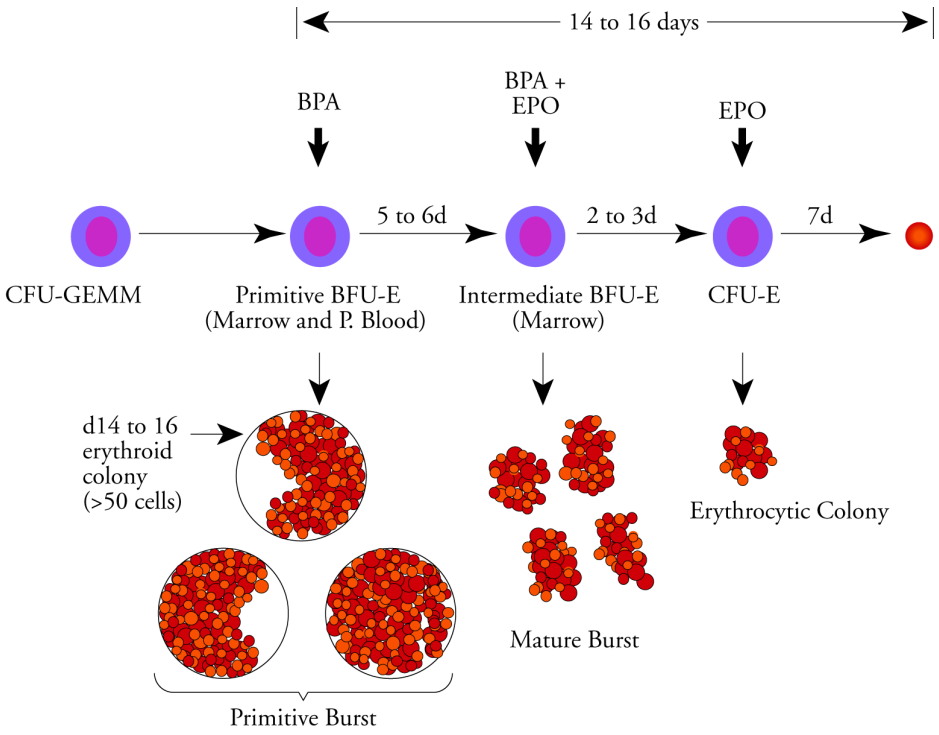

Fig. 3 - Diagram of the stages and colony morphology of erythropoiesis in tissue culture, from the myeloid multipotential CFU-GEMM to mature RBCs.

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Ferritin is a glycoprotein produced by phagocytes of the reticuloendothelial (RE) system, and involved in supplying iron to tissues. Its variants are tissue-specific. Ferritin, like hemosiderin, is also an iron storage molecule - in hepatocytes, and in macrophages of marrow and spleen. Low ferritin concentration in anemia signals an iron deficiency, whereas a high concentration of ferritin in anemia signals instead iron overload, typically one associated with excessive destruction of RBCs (hemolysis). In malignancy, low serum ferritin suggests that an iron deficiency is superimposed on anemia of chronic disease (see below), whereas a high serum ferritin suggests hemolysis associated with B cell chronic lymphocytic leukemia (B-CLL), Hodgkin's disease, acute leukemia or gastro-intestinal carcinomas. **Table 1** shows the differential iron diagnostics for anemia and cancer.

The last group of microcytic anemias encompasses deficiencies in iron transport, utilization or re-utilization.

Iron transport defects (rare) are congenital and due to either lack of transferrin or defective transferrin, and thus called atransferrinemias. Transferrin, a glycoprotein, is also a serum transporter

Table 1
Differential Iron Diagnostics

		Elisa	R.I.A.	RIA	
	transferin binding capacity	serum transferin receptor	serum Ferritin	RBC Ferritin	Free RBC proto-prophyrin
Iron-deficiency anemia	↑	↑	↓	↓	↑
Anemia of chronic disease	↓	Normal	↓		↓
Hemolytic states			↑		
Fe-overload syndromes			↑	↑	
Hepatitis			↑	N	
Acute leukemia			↑	N	
Hodgkin's disease			↑	N	
GI cancer			↑	N	
Iron-Transport-Deficiency anemia	↑		↑		

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of iron - one that is employed by erythroblasts and hepatocytes for the specific intake of the iron-transferrin complex via endocytosis. Atransferrinemias result in poorly hemoglobinized microcytes.

In patients with iron utilization anemias there is inadequate utilization of iron for hemoglobin (Hb) synthesis, despite adequate serum and intracellular concentrations of iron. These anemias are, therefore, hemoglobinopathies, primarily of the thalassemic type (see below). Sideroblastic anemia - characterized by the presence of sideroblasts in peripheral blood - is often classified as a microcytic anemia of the hemoglobinopathic type. However, sideroblastic anemia is best understood as a symptom of late MDS (see below), and since, aside from siderocytes and hypochromic microcytes in peripheral blood (and ringed sideroblasts in marrow), it is mostly normocytic, it is briefly treated below under the rubric of normocytic anemias.

Anemia of chronic disease (see **Table 2**) is thought to be caused by the unregulated action of inflammatory cytokines, whether due to persistent inflammation, infection or cancer. Anemia of infection - a variant of anemia of chronic disease - is treated below in its own subsection. In anemia of chronic disease, the intracellular iron metabolism is fundamentally impaired because bone marrow reticulum cells that retrieve iron from senescent RBC's, fail to release it for Hb synthesis. The result is failure to respond with erythroid growth to the anemia, and thus reticulocytopenia ensues. Most often the anemia is "mild" (not <8g/dL of Hb) and responds to treatment with rhu-EPO and Fe supplements. However, refractory marrow response to EPO is observed in cancer patients with anemia.

Table 2
Anemia of Chronic Disease

	Chronic Inflammation	Chronic Infection	Cancer
1. Shortened RBC Survival or $^1/_2$ life	-	Granulomatous disease	+
2. Decreased EPO production	+	+	+
3. Impaired intracellular Fe metabolism	+	+	+

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1.1.2. Normocytic Anemias: hypometabolic, aplastic and refractory

Deficient erythropoiesis is the direct cause of all normochromic-normocytic anemias, and it may be rooted in various and distinct factors:

1. Relative or absolute decrease in EPO production
2. Hypometabolic state (of erythroid progenitors) that fails to respond to EPO
3. Loss of erythrocyte precursors (eg in aplastic anemia) due to
    - 3.1 defect in stem or progenitor cells
    - 3.2 injury to marrow environment
4. Replacement of marrow by a neoplasm, fibrosis or sclerosis

With exception of the first entry, all other factors involve some degree of generalized bone marrow failure. Sideroblastic anemia and myelodysplasia aside, the other normochromic-normocytic anemias are characterized by normal RDW (RBC distribution width), reticulocytopenia, and a failure of the erythroid compartment to expand (hypoplastic state).

Hypoproliferative anemias may be due to renal disease - mostly glomerular lesions - that adversely affects the production of EPO, the main normal hormone or cytokine (glycoprotein) driving erythropoiesis. However, as we shall stress repeatedly throughout the present essay, EPO is not the only cytokine involved in driving erythropoiesis, nor is it even necessary for normal erythropoiesis - at least not for primary marrow or PB cells grown in serum-free tissue culture. Thus, the fact that in such hypoproliferative anemias no erythroid hyperplastic response is observed - in particular one driven by one or more of the other erythropoietic cytokines - suggests that more than deficient EPO production or an hypometabolic EPO response may be involved. In light of the Correa-Axelrad model of erythropoiesis [1] (see below), the absence of a reacting erythrocytosis in these anemias also suggests an hypometabolic IGF-I response, which may likely be just a component of a general hypometabolic state that would include hypothyroidism and hypopituitarism. In this context, it is noteworthy that hypoproliferative anemias are also associated with poorly defined protein depletion disorders and uremia.

In aplastic anemia, one encounters either what is called a "pure RBC aplasia" characterized by marked reduction of erythroid precursor cells and attendant erythropenia, or, more frequently, a pan-hypoplasia of the marrow that also presents leucopenia and thrombocytopenia. Pure RBC aplasia has been diagnosed following not just exposure to toxins (organic phosphates), or drug intake (tranquilizers, anticonvulsants), but also - and more importantly from our viewpoint - to viral infections (human parvovirus), or as a result of malignant disease, in particular, thymomas and B-CLL. In the latter, it may well be the result of an auto-immune reaction against erythrocytes and their antigens.

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RBC production decreases as a result of the erythroid hypoplasia. RBC's produced are normochronic-normocytic. Reticulocytes are at first decreased, then become absent as the erythroblastopenia progresses. If pure red cell aplasia is associated with some degree of marrow hypoplasia, it is accompanied by a severe thrombocytopenia, and bleeding into skin and mucous membranes, including the ocular fundi. No splenomegaly is observed since the condition results in a circulation deficit of RBCs. The bone marrow tends to become increasingly acellular, and the susceptibility to infection is life-threatening. Treatment with EPO is often tried, but marrow response depends on whether there are any erythroid precursors left that can be recruited. Bone marrow transplantation (from an identical twin or HLA-compatible sibling) is the preferred conventional treatment, since it will seed new stem and erythroid progenitor cells. However, if the etiology of the aplasia is auto-immune, marrow transplantation will not "take".

Indeed, of particular interest to our study is that a significant percentage of patients with pure RBC aplasia can be "successfully managed" with immunosuppressants - either corticosteroids like prednisone, or toxic immunosuppressant agents like cyclosporine. This suggests that a RBC-specific auto-immune response underlies the pure RBC aplasia, and the relationship is made evident when the aplasia is associated with thymoma and these patients improve upon thymectomy. When RBC aplastic anemia is associated with generalized marrow hypoplasia that results in pancytopenia, it should be called "aplastic pancytopenia", rather than "aplastic anemia". In thymoma-induced aplastic pancytopenia, the treatment consists of thymectomy and administration of equine antithymocyte globulin.

"Aplastic pancytopenia" often presents anisocytosis with mild macrocytosis of PB RBCs. Recognized causes of "idiopathic aplastic anemia" - ie "aplastic pancytopenia" - are environmental or pharmaco-iatrogenic: chemicals such as benzene and other solvents, or arsenic, mercury and other heavy metal ions; ionizing radiation; medical drugs (cancer chemotherapeutics, antibiotics, NSAIDs, anti-convulsants). The mechanism is thought to be, in general, selective hypersensitivity, suggested as genetic in nature. There is little doubt that some aplastic anemias are familiarly acquired or genetically determined, such as Fanconi's anemia or the rare congenital Blackfan-Diamond anemia (DBA), and only diagnose upon onset of subsequent inflammatory or infectious disease. But it seems that rather than selective, or genetic, hypersensitivity to chemical toxicity, it is more appropriate to speak in terms of degrees of sensitivity to toxicity, and degrees or intensity of exposure. Since onset is most frequently insidious, and the causation multiple, the best approach is to describe the vector concatenating the symptoms - one of which is anemia. At any rate, a sizable portion of the pure RBC aplasias and the "aplastic pancytopenias" are acquired auto-immune disorders.

Other causes of deficient erythropoiesis that results in normocytic anemia relate to the dis-

 ISSN 1920-3799

ruption of bone marrow structure and function, typically as the outcome of its replacement by a neoplasm, fibrosis or sclerosis. Marrow infiltration by a neoplasm - viz. lymphoma, myeloma or a metastatic carcinoma - is often referred to as myelophthisic anemia. The most common cause of myelophthisic anemia is therefore thought to be metastasis from primary tumors to the marrow, resulting in marrow infiltration by tumorigenic cells. Conversely, replacement of marrow by fibrosis is a consequence of the hyperplastic growth of marrow cells, or myeloproliferation. The resulting anemia is referred to as myelodysplastic anemia. Myelophthisic and myelodysplastic anemias are types of refractory anemia. Their conceptual distinction, however, may not be a precise one. On one hand, there is ample reason to treat myelodysplastic anemia as a neoplastic disease, while, on the other hand, marrow lymphoma may also be a neoplasm endogenous to marrow. Most importantly, myelodysplastic anemia appears to be one of the symptoms of a more encompassing neoplastic disorder, often and indistinctly called myelofibrosis, agnogenic myeloid metaplasia, myelosclerosis with myeloid metaplasia, or myelodysplastic syndrome (MDS). Myelodysplasia - or, more properly, the complex of "myelodysplastic syndromes" that includes CMML (MDS, see **Table 3** and sections 5, 6 and 7 below) - connotes a myeloproliferative disorder characterized by progressive bone marrow failure in the absence of overt blast cell proliferation. Because of the absence of blastosis, MDS fails to fit the criteria for AML (acute myelogenous leukemia), even though 40 to 60% of myelodysplasia cases evolve into AML. But MDS is not, like aplastic normocytic anemias, a *hypo*proliferative disorder, even if some patients show a hypoplastic marrow at presentation. Rather, MDS is pre-acute hyperproliferative disorder that may be regarded as having a leucoerythroblastoid crisis before evolving into acute leukemia. Technically, MDS is said to include "preleukemia" (an ill-defined condition that generally means pre-acute leukemia; see discussion in sections 5 and 6 below), the so-called refractory anemias (including sideroblastic anemia), Ph-negative CMML (which presents absolute monocytosis), erythremic myelosis and myelofibrosis.

We will examine this myeloproliferative disorder more at length below in a separate rubric, but presently we want to emphasize the distinct aspects of early and late MDS. Early phases of the disease are characterized, as we said, by marrow hyperplasia with a predominance of erythroid progenitors and by a normochromic anemia coexisting with metaplastic foci in the spleen and elsewhere [16-18]. In marrow, normal or increased rates of erythropoiesis are observed, but overall the lifespan of RBCs is reduced, and pronounced morphological changes - hypochromia, anisocytosis, poikilocytosis - are encountered. Erythrophagocytosis by macrophages is often ongoing and lipid storage in marrow stromal cells abnormal. In peripheral blood, nucleated, immature RBCs are frequent (mostly normoblasts, but also reticulocytes). Splenomegaly is most often the presenting symptom, along with hepatomegaly. The spleen is enlarged by the myeloid metaplasia (tissue conversion), and the extent of

 ISSN 1920-3799

Table 3

Classification of myelodysplastic syndromes on the basis of morphologic criteria.
After Sarkodee-Adoo et al [160].

Syndromes	% cases	% blasts in PB MNC	Median Survival (mo)
RA	30%	<5%	50
RA with sideroblastosis (RARS)	20%	<5%	50
RA with excess blasts (RAEB)	20%	<20%	11
RA with blast transformation (RAEB-T)	15%	<30%	5
CMML	15%	<20%	11

the splenic extramedullary hemopoiesis is such as to duplicate the appearance of normal bone marrow. Subsequently, the disorder evolves into myelofibrosis with megaloblastoid cells and binucleate erythroblasts, with the aspect of an erythroblastic leukemia not too far from acute erythroleukemia. Progressive myelofibrosis disrupts marrow sinusoids and shuts down marrow hemopoiesis; partial or total myelosclerosis (accompanied by new bone formation) may follow, or, even more frequently, osteoporosis.

The late myelodysplastic pattern of symptoms is often called "leukoerythroblastic", and it may be encountered with a WBC count that is normal, reduced or, in the majority of instances, increased. Since the marrow is progressively destroyed and the myeloid metaplasia is mostly dysfunctional, patients only present at best, modest responses to EPO and other regulatory cytokines. The biological significance of the extramedullary hemopoiesis remains controversial. Early views stressed the compensatory nature of the myeloid metaplasia as a response to marrow failure, whereas later schools of thought have emphasized it as a basic trait of myelodysplasia. In 1951, Dameshek proposed that marrow cells - in particular erythroblasts, granulocytes, monocytes and megakaryocytes - develop, together with fibroblasts, as a formative unit [19]. This went a long way towards explaining the multiple symptoms associated with the great majority of hematologic neoplasms. But since prolifer-

 ISSN 1920-3799

ating fibroblasts are not derived from the pluripotential stem cells that give rise to the myeloid lineages, the fibroblast hyperplasia observed in the marrow and spleen of MDS patients could constitute either a response to the myeloid hyperplasia, or a primary proliferation driving the latter. The functional marrow hematopoietic unit (hematon) has since been identified - both by separation and tissue-culture methods - and it is virtually absent in MDS, CML and AML [20]. This suggests that the hyperproliferation of both myeloid and connective tissue cells observed in MDS is directly related to the disturbance of the structure of the hematon and its coordinated regulatory functions. It is noteworthy, in this context, that while Polycythemia *vera* may progress towards myelofibrosis, the latter frequently evolves towards erythroleukemia and other forms of acute myeloid leukemia (see below). These conversions put into evidence the neoplastic nature of myelofibrosis/myelodysplasia and the associated refractory anemias - and, in our view, raise the question of what we have proposed under the rubric of "an acquired auto-oncogenic vector of disease progression" [1]. Since MDS affects all myeloid lineages, it likely involves a complex of disorders of a myeloid stem cell, on a footing comparable to that of chronic myeloid leukemias when it comes to the potential to evolve into acute leukemias. Current thought is that MDS consists of a multiplicity of clonal stem cell myeloproliferative neoplasms, with variable hemopoietic emphasis. However, given the involvement of all myeloid lineages, it is unlikely that the stem cells involved are hit at a stage more primitive than the CFU-GEMM. If this proves to be the case, then blast cell disorders must hit a more primitive myeloid progenitor, likely of the CFU-S type.

Frequently, myelodysplasia progresses to, or is found associated with, ringed sideroblasts in marrow and siderocytes and target cells in peripheral blood. Sideroblasts are normoblasts with a cytoplasm that contains non-heme iron granules normally not observed in erythroid precursors, since mitochondria compartmentalize toxic iron (and sulfide) away from the cytosol as part of their central role in the biosynthesis of iron-sulphur clusters [21-22]. When marrow is stressed, RBCs may form these granules and mature without having eliminated them first, whenever they fail to export ferritin. These RBCs are termed siderocytes. But in so-called sideroblastic anemia or refractory anemia with ring sideroblastosis (RARS) (see **Table 3**), the non-heme iron deposits instead inside the mitochondria, between the cristae. The perinuclear ring-like disposition of mitochondria then causes the characteristic appearance of the ring (or ringed) sideroblast. It is thought that sideroblastic anemia may be primary when due to defective anabolism of heme and hemoglobin in erythroid precursor cells [23]. However, it is most commonly associated with MDS, hemolytic anemia, leukemia and myeloproliferative disorders. The EPO secretion is normal in RARS patients, and ca. half of the patients respond to it but require pharmacologic doses [24].

From the preceding, it appears that, with the exception of nonEpo-dependent "pure RBC

 ISSN 1920-3799

aplasia", all the other forms of aplastic and refractory anemias involve hematopoietic defects that are not limited to impact solely the erythroid compartment. It would, therefore, be best to consider them as symptoms of myeloid and connective tissue proliferative disorders, rather than properly erythropoietic disorders (or, strictly speaking, anemias). While aplastic anemias would be hypoproliferative, it seems that refractory anemias should properly belong to the category of hematologic neoplasms.

1.1.3. Macrocytic Anemias:

MDS and vitamin deficiencies in pernicious anemia

Macrocytic anemias are generally megaloblastic, ie display macro-ovalocytes in peripheral blood. Nonmegaloblastic macrocytic anemias are poorly understood, often presenting an excess of RBC membrane (eg chronic liver disease, chronic alcoholism) in the absence of a megaloblastic marrow. Oval macrocytes in megaloblastic disease are associated with defective DNA synthesis, and an increase in cytoplasmic mass that results from unregulated RNA synthesis. Megaloblastoid RBC precursors are present in marrow - and released to the circulation - but are the outcome of disturbed hemopoiesis affecting all myeloid lineages. Accordingly, the megaloblastic anemia is typically accompanied by leukopenia and thrombocytopenia. The extent to which the two marrow scenarios of macrocytic megaloblastic anemias - the megaloblastoid and pancytopenic phases - are distinct from the leukoerythroblastic and sclerotic phases of myelofibrosis or MDS is unclear, were it not for the absence of marrow fibrosis.

Iatrogenic causes of megaloblastoid macrocytic anemia include toxic immunosuppressants and cytotoxic drugs used in cancer chemotherapy. Critically, other common causes of megaloblastic anemia include specific vitamin deficiencies - folic acid, vitamin B_{12} (cyanocobalamins) and vitamin C. Macrocytic anemias have therefore an heterogenous etiology, but their commonality lies in the fact that exposing marrow to cytotoxic agents disturbs erythropoiesis in a manner equivalent to depriving it of vitamins, nutrients or growth factors.

The most frequently encountered cause of megaloblastic anemia is vitamin B_{12} deficiency. In the classical form of "pernicious anemia" caused by this deficiency, marrow megaloblastoid cells result from a specifically erythroid hyperplasia that is unable to generate normal, mature differentiated RBCs. In peripheral blood smears, the symptomatic macro-ovalocytes coexist with poikilocytic RBCs and morphologically abnormal large platelets. Cytoplasts and pyknotic nucleus fragments (Howell-Jolly bodies) are frequent, and serum lactate dehydrogenase A (LDHA) is elevated - all indications of increased hemolysis. Thus, pernicious anemia caused by B_{12} deficiency evolves into an hemolytic anemia. It is also associated with a significantly increased risk of gastrointestinal carcinomas. B_{12}, an essential cofactor in nucleic acid metabolism, can only be absorbed by the small intestine if complexed with intrinsic factor, a secretion of the stomach's parietal cells. The term pernicious anemia techni-

 ISSN 1920-3799

cally refers precisely to loss of this intrinsic factor. 80-90% of the patients with pernicious anemia show auto-antibodies against the parietal cells, most antibodies being specific for the intrinsic factor. Thus, the majority of the B_{12} anemias appear to have an auto-immune etiology. This constitutes a seldom remarked vicious spiral - that most of these anemias have an auto-immune basis which results in RBC hemolysis, and that this hemolysis in turn stimulates further auto-immune reactivity that may target other antigens (eg those in RBCs), rather than intrinsic factor or parietal cell antigens. The spiral's result is to further intensify both the anemia and the hemolysis.

Yet, as we said, the etiology of B_{12} anemias is far wider than its majoritary auto-immune component. Indeed, it can be brought about simply by inadequate diet or nutritional intake of B_{12}, which, as a supplement, is best administered in time-release preparations. Lack of B_{12} may even be just the result of competition for the vitamin by intestinal fish-tapeworms or bacterial parasites - as in the "double loop syndromes". Or the lack of B_{12} may be relative, despite a normal intake, due to conditions that increase its requirement, such as hyperthyroidism or alpha-thalassemia. Inadequate absorption of B_{12} may also be simply the result of a lack of intrinsic factor production. In this context, the possibly iatrogenic role played by the popular proton-pump inhibitors -prescribed to "treat" acid reflux - in promoting lack of intrinsic factor production has not been adequately studied. Pancreatic disorders may preclude expression of enzymes needed by the small intestine to liberate B_{12} from the binding proteins presented in saliva, in order to permit B_{12} to bind in turn to intrinsic factor. Similarly, deficient binding to serum-transporter proteins - in particular to transcobalamin II - as is often seen in liver and kidney disease, will result in increased excretion of B_{12} and a relative lack of it despite intake in the normal range.

Pernicious anemia caused by a B_{12} deficiency is also accompanied by neurodegenerative disease, affecting at first myelinated peripheral nerves and cerebral white-matter, and progressing to corticospinal tract and cortical neurons. B_{12} serves as cofactor in myelin formation, and thus the effects of neural degeneration mimic symptoms of multiple sclerosis and compressive cord injury, from which it must be differentiated. Intensification of the B_{12} deficiency leads to delirium and spastic ataxia. Thus, B_{12} deficiency is a multi-system disease manifested by inefficient erythropoiesis, that eventually develops into dyspoietic and neurodegenerative disorders.

Megaloblastic anemia due to folic acid deficiency (once called vitamin B_C or *Citovorum* factor, in the enzymatic co-factor form that synergizes with vitamin C) parallels in nearly all respects, save one, the pernicious anemia caused by B_{12} deficiency. The major difference is that it does not present neurological lesions. However, B_{12} deficiency itself promotes folate deficiency by decreasing folate utilization [25]. Thus, another negative feedback loop that enters into a spiral of increasing hematological disorder may be associated with the classical pernicious anemia. This is further aggra-

 ISSN 1920-3799

vated by lack of vitamin C, a cofactor in the absorption and utilization of folic acid. Accordingly, folate deficiency and resulting megaloblastic anemia are typically observed in scurvy patients. The intimate relation between the two megaloblastic anemias - due to B_{12} and folate deficiencies - is further underlined by the greatest clinical caution against treatment of folate deficiency without prior ascertaining of whether there is an underlying B_{12} deficiency: treatment of the anemia with folic acid without ruling out B_{12} deficiency may result in acute neurological damage caused by immediate aggravation of the B_{12} deficit.

Inadequate folate nutritional intake or absorption are the most frequent causes of folate deficiency. The nutritional insufficiency may be simply due to dietary lack of fresh vegetables, since most cooking times and temperatures destroy folates, or due to impaired hepatic function, as caused by chronic alcohol use. Cytotoxic drugs, oral contraceptives and barbiturates decrease folate absorption.

Folates are coenzymes needed for nucleotide synthesis and amino acid conversion. They are essential for proper hematopoiesis, and their requirement increases whenever hematopoiesis is accelerated, whether as a normal response (in pregnancy, lactation, development, etc), or as a consequence of an underlying clinical condition (psoriasis, B_{12}-dependent pernicious anemia, beta-thalassemia major, etc). As with classical pernicious anemia, anemia induced by lack of folate operates as a vicious spiral - lack of folate, relative or absolute, stimulates hemopoiesis, but thereby also condemns the latter to dyspoiesis; the resulting megaloblastoid cells are fragile and hemolyze fast, increasing the demand for hemopoiesis and aggravating further the lack of folate.

The importance of vitamin C in respiratory metabolism and the maturation of lymphocytes has already been discussed in the previous communication [1]. However, lack of vitamin C has another major consequence - in that it directly affects erythropoiesis. Typically, this is manifested by the presence of circulating hypochromic RBCs of normal size. If the lack of vitamin C coexists with an iron deficiency, then hypochromic RBCs are microcytic, and if it coexists with a folate deficiency, then circulating macro-ovalocytes are observed.

1.2. Hemolytic Anemias

The term hemolysis is unfortunately applied to designate both a 'leaky' state of the RBC and the end point of its physiological degradation. Further unfortunately, hemolysis also designates a specific process associated with that state, but which is not the only way that a RBC degrades or lyses. The state designated by the term is a dysfunctional one, characterized by the RBCs becoming leaky and releasing hemoglobin. If these RBCs were not subject to removal by the spleen (testing, filtration, phagocytosis), they would leak all of their cytoplasmic contents to blood and become ghosts (empty membrane envelopes). The normal function of the spleen is thus to remove from circulation RBCs found to be in the hemolytic state, ie in an hemolytic process. And that's why the process of hemol-

 ISSN 1920-3799

ysis is also used to denote and encompass the phagocytic action of spleen macrophages, as when it is said that "most hemolysis occurs outside the circulatory system, through the action of phagocytes in spleen, liver and marrow".

To make matters worse, hemolytic anemia is frequently defined as a disorder characterized by precocious senescence of RBCs. Obviously, in a wider sense, the end point of virtually every anemia must be 'hemolytic', since anemia is always associated with a defect of RBCs, and every such defect implicates a precocious senescence of RBCs. In anemias that are considered nonhemolytic (iron deficiency, lack of response or altered response to regulatory cytokines, underlying malignancy or vitamin deficiencies), such defects produce various kinds of abnormally shaped RBCs that are also removed from circulation by the action of the spleen.

What characterizes the group of the hemolytic anemias is that it gathers together various processes that induce the relatively rapid lysis of RBCs by the loss of hemoglobin across leaky membranes. Hemolysis may be caused by RBC independent factors or by RBC dependent factors, which include both intrinsic defects and auto-immune reactions. RBC independent factors are encountered in mechanical trauma, poisoning or infection. RBC dependent factors, of greater interest to our disquisition, involve a variety of distinct pathways. However, some forms of chemical poisoning matter to our inquiry, not just because they are essentially poisons of cellular respiratory metabolism, but also because they are or may be common enough as to constitute a covert stimulus for dyspoietic formation of RBCs - and thus the ultimate cause of this or that 'hemolytic anemia', or "what got the ball rolling to begin with". This is the case, for instance, of copper, in particular for low level poisoning with it. Of greater interest to our discussion of proliferative disorders of the blood system are the auto-immune forms of hemolysis, because they may well underlie the neo-lamarckian evolution of dyspoietic blood disorders into full-fledged blood neoplasms.

In our presentation of the hemolytic anemias, we will not follow the usual distribution and classification. We think that autoreactive anemias - effectively the main group - should be coupled together, and that these disorders should be understood in the context of our proposed vector of auto-oncogenic disease [1]. This view will be the focus of the follow-up communication, inasmuch as the hemolysis observed in the hemolytic anemias detailed here is not the only form of RBC degradation or lysis (decomposition) associated with anemia (or anemias) or with the auto-oncogenic vector that we have proposed [1]. A process somewhat analogous to apoptosis - or more correctly, parallel to the progressive self-excisions of cytoplasmic fragments observed in the autoschizis of nucleated cells [26] - has been described for RBCs. Like apoptosis, it involves phosphatidylserine (PS) externalization, hence has been termed 'eryptosis' [27-29]. Specifically, it is our suggestion that this form of RBC degradation that results in the vesiculation or fragmentation of RBCs entails a crenation vector for the pro-

 ISSN 1920-3799

duction of echinocytes and acanthocytes that may invoke auto-immunity.

1.2.1. Auto-immune Anemias (AIA)

We will include under the AIA entry, both phagocytic disorders of the reticuloendothelial system, and the classical AIHA (autoimmune hemolytic anemia) that is closely associated with Chronic B cell Leukemias (CLL in particular) and should rather be called auto-immune *humoral* hemolytic anemia (AIHHA).

We should note that, as mentioned above, hemolysis-causing erythrophagocytosis is also pronounced in refractory anemias, and that hemolysis is observed in pure red cell aplasia, aplastic anemias and pernicious anemias, where it also implicates diverse auto-immune reactions. In other words, there are anemias with different symptomatologies and etiologies which share, nonetheless, the hemolytic outcome and involve auto-immune responses, whether cytotoxic or humoral. These facts have suggested to us that there may well be an insight in regarding, the way Wilhelm Reich did [30-31], auto-immune hemolytic anemia as one of the main driving forces in the evolution of the process of auto-oncogenic disease - from the debility or weakness of the red cell compartment to the flaring up of full fledged leukocytic or leukoblastic leukemias. In other words, it is increased membrane lability or precocious senescence (whatever the various factors involved) of RBCs that stimulates the immune system - whether phagocytic or humoral, or both at the same time - to react against an increasing pool of unviable and fragmenting RBCs and induce auto-immune anemias. In the process of autoreaction, an expansion of leukocytes must occur, whether myelocytic or lymphocytic, or both, and this is what constitutes the overt leukemia, secondary to both the regulatory disturbance and the increased fragility and dysfunctionality of RBCs.

1.2.1.1. Phagocytic AIA:
hypersplenism or congestive hypersplenism

Hypersplenism or congestive splenomegaly is most frequently caused by a myeloproliferative disorder, an hyperplasia of the phagocytic component of the reticuloendothelial (RE) system, and it targets mostly RBCs, but frequently also platelets and various circulating WBCs (often with a normal WBC differential). Thus, it is likely that the root of the auto-phagocytic reaction is some form of a detectable myeloid dyspoiesis. The resulting anemia is typically mild and frequently undetected. Eventually there is a trauma-like mechanical component - a congestive effect - to hypersplenism, but from the onset, and without any mechanical trauma being present, there is both an increased phagocytosis of RBCs and an increased RBC filtering (a mechanical sieve-like action) by spleen macrophages. We will suggest, in this and the next communications, that it is the fragility, weakness and inflexibility (rigidity) of circulating RBCs that serves as stimulus for the initial hypersplenism (or auto-reaction). Simply put, the RE system - likely together with the lymphocytic system - detects that

 ISSN 1920-3799

something is deeply wrong with the circulating RBCs and mounts an attack on them. This argument is strengthened by the frequent association of hypersplenism and erythrophagocytosis with AIHA [32-33]. In fact, peripheral blood erythrophagocytosis by both macrophages and neutrophils is one of the factors considered to be indicative of AIHA.

Splenomegaly results from the contribution of various vectors - the hyperplasia of RE phagocytes concomitant with the autoreaction, the increased phagocytic and mechanical removal of RBCs, and the increased sequestration of RBCs in the splenic space which results in congestion. The consequences of hypersplenism are therefore extreme - peripherally, in circulation, there is a growing cytopenia that affects not only the red cell compartment, but also other myeloid compartments (granulocytes and platelets); whereas, in bone marrow, increased cellularity from hyperproliferation of the myeloid precursors of the affected compartments is observed.

It is rather likely that disturbed autoreactive phagocytosis directed above all against the RBC compartment is mediated by an altered interaction of T cells - in particular T-helper and T-cytotoxic cells - with members of the RE system, likely antigen-presenting cells (APCs) of the type of the Langerhans macrophage. The sites of this altered interaction will be lymph nodes. It is possible that the disturbance may affect marrow erythropoiesis and also form a self-feeding loop, since marrow macrophages are known to regulate erythropoiesis [34].

Given that the disorder is generally considered to be strictly one of the RE system, so that the hemolytic anemia is solely produced by splenic sequestration, it is thought not to have associated RBC morphological changes. It is possible that an altered membrane or cytoskeletal function may not yield a gross morphological change, but the suggestion we make - and have made above - is that, in all likelihood, the disorder is first caused by a covert or overt defect in RBC function that fragilizes the red cell and serves as stimulus for the autoreactive response of the RE system. We will explore the possibility this may actually be an overt and morphological set of changes in the accompanying communication.

1.2.1.2. Auto-immune humoral hemolytic anemia (AIHA, or rather AIHHA)

As of 1908, it was thought that the etiology of some instances of acquired hemolytic anemia may be auto-immune. By the 1930's, it was clear that auto-immunity was responsible for acute manifestations of hemolytic anemia. AIHA is today identified as an auto-immune disease caused by B cells producing auto-antibodies against RBCs. These autobodies are detected by the Coombs' (direct antiglobulin) test, but their presence alone is not sufficient to cause hemolytic disease. Two types of classical AIHA exist, warm antibody disease and cold antibody disease. Warm-antibody AIHA is found in association with a variety of neoplasms, particularly malignant lymphoproliferative disor-

 ISSN 1920-3799

ders, or as a primary manifestation with no apparent cause (which is the meaning of the term idiopathic).

Warm antibody AIHA has sudden onset, presents slight splenomegaly and the anemia is severe and progresses rapidly. Spherocytosis is acute with high MCHC, and polychromatophilia soon develops. The warm-reacting auto-antibody is found bound to RBCs at 37°C. B cells appear to express them when altered, or perhaps even transformed, by malignant disease - as in CLL, B cell lymphoma or *Lupus erythematosus*. Remarkably, dopamine use appears to promote anti-RBC warm auto-antibody expression. Different patterns of reactivity exist involving both IgG and complement C3. Most of the auto-antibodies are panagglutinins directed against the Rh antigen.

Cold antibody AIHA only reacts at temperatures between >30° and <37°. It is typically associated with infections, whether ordinary bacterial infections (eg *Haemophilus influenzae*, *Streptococcus pneumoniae*), or mycoplasmal infections (eg with *Mycoplasma pneumoniae*, Ureaplasma), or still viral (eg infectious mononucleosis with EBV) and even protozoal (eg *Giardia lamblia*); and it is also associated with proliferative lymphoid disorders (typically CVI, common variable immunodeficiency) that most frequently constitute the fertile ground (the depressed immune state) for opportunistic infection with a bacterium or a virus [35]. Cold auto-antibody AIHA may be accompanied by ITP (idiopathic thrombocytopenic purpura) .

Thus, AIHA is not an autonomous entity. It forces us to think through the complex causes that underlie its expression. Acquired AIHA is not necessarily due to any particular infection or toxicosis, even though it is well established that alpha-methyldopa and a wide range of drugs can induce AIHA. Specifically, what underlies most acquired AIHA appears to be a lymphoid disorder that is stimulated or promoted by an auto-immune reaction against RBC antigen determinants. The fact that almost all advanced CLL patients present AIHA underscores this view. Moreover, normal B cells constantly generate auto-immune antibodies, but these cells are the object of a T cell suppressor activity that represses auto-antibody expression. It is a disturbance of the T suppressor activity that permits autoreactive clones to amplify and express humoral auto-immunity [36].

At the root of AIHA there is then a defective regulation of T cell control of idiotypic networks [37] that results in humoral autoreaction . In this context, our explicit suggestion is that, if we go one step further back in the etiology of AIHA, we will find that *this T cell dysregulation is an adaptive immune response to the increased dysfunctionality of the RBCs produced and set into the circulation*. The RBC dysfunction may be due to deficient erythropoiesis, dynamic causes (chronic hypoxia being foremost), or in most cases a combination of both. Irrespective, we propose that it is the greater potential for hemolysis of the circulating RBCs that eventually triggers the T cell dysregulation, lifting the block to the autoreactive B cell clones and magnifying hemolysis to the point of splenomegaly

 ISSN 1920-3799

and anemia.

The proposed view comes at the encounter of what Dacie proposed in 1992 [38]. Dacie etiologically separated the AIHAs into acute transient AIHA of infectious origin, chronic AIHA and chronic cold-haemagglutinin disease (CHAD). The main form, chronic AIHA, is the one of interest in the present context, since Dacie suggested that its etiology stemmed from the breakdown of self-tolerance, "perhaps by a failure of T-lymphocyte surveillance or an abnormal T-suppressor to T-helper ratio". Dacie also indicated that "immunoglobulin abnormalities, particularly IgA deficiency, appeared to play a part in the breakdown of tolerance" [37].

If, technically, the lymphocytic leukemia or chronic neoplasm (eg CLL) consists exactly of the growth and expansion of the auto-reactive B cell clone (even if this proliferation goes under the radar because it does not immediately result in overt PB leukocytosis), then, in the context of our suggestion, one arrives at the oncogenic vector of acquired AIHA, where the hemolytic anemia is the result of a leukemia, and the leukemia an adaptive response to the dysfunctionality of the RBCs:

increased RBC lability —> T suppressor dysregulation —> anti-RBC autoreactive B cell clone —> AIHA

This fits with the discovery by Rosenthal et al in 1955 that most of the AIHA patients had overtly suffered from CLL or Hodgkin's disease [39-40]. According to our proposal, it is the acquired RBC defect or defects that initiate the auto-oncogenic process, by eventually launching a humoral autoreaction responsible for acquired AIHA. Moreover, it is the humoral reaction (causative of anemia) that gives rise to a leukemia during the amplification of the autoreactive clone, and not the other way around, not the anemia that leads to leukemia; thus *the hemolytic anemia is already leukemic*, even when no overt leukemia is detected. What potentially leads to leukemia is the initial RBC lability, which encompasses a variety of factors explored further in the next communication, but which come down to the inability of RBCs to perform their function of capturing and delivering oxygen to the tissues.

Moreover, the proposed sequence of the auto-oncogenic vector schematically shown above may well not be specific to AIHA, since it may underlie hypersplenism and be an integral part of certain anemias caused by erythropoietic defects - as in pure RBC aplasia or in the *refractory anemias* during the erythrophagocytic and sideroblastic phases when hemolysis intensifies (see section 5 below). Furthermore, a parallel dysregulation of T suppressor response in response to vitamin B_{12} deficiency could well explain the anti-parietal autoreactive B cells responsible for *pernicious anemia* (again, the anemia would be the result of an auto-immune disorder). Thus, in light of all this and from a functional perspective, the AIA category should also include some technically normocytic and

 ISSN 1920-3799

megaloblastic anemias.

In contra-distinction to this, we should keep in mind that complement-sensitive hemolytic anemia (Marchiafava-Michell syndrome, see next subsection) is not, strictly speaking, auto-immune but the result of an acid-sensitive (checked by Ham's acid hemolysis test) plasma membrane lability caused by an X-linked biosynthetic defect responsible for producing an abnormal PI (phosphoinositol) anchor. The defective anchor fails to retain transmembrane proteins. Once again, we must underline how hematologic disorders that present the same or analogous symptoms may have very different etiologies which must be separated according to whether they are genetic (innate), auto-oncogenic (adaptive), infectious, etc.

1.2.2. Hemolytic anemias caused by RBC membrane disorders

In general, hemolytic anemias caused by RBC membrane defects are either hereditary, and thus congenital, or acquired. They stem from altered protein structures of the complex RBC cytoskeleton that cortically anchors the plasma membrane and gives the cell its doughnut shape.

Hereditary disorders include the Marchiafava-Michell syndrome, hereditary spherocytosis (HS) and hereditary elliptocytosis (HE). The Marchiafava-Michell syndrome typically is manifested in early adult males (the result of a single hit) as a normocytic anemia, and accompanied by leukopenia and thrombocytopenia. Often it progresses towards marrow hypoplasia and aplasia. HS is an autosomal dominant trait characterized by production of fast-hemolyzing spheroidal RBCs, presenting splenomegaly and jaundice. Several alterations of membrane bound proteins result in the spherocytic phenotype. The rare HE is also an autosomal dominant trait affecting the RBC cytoskeleton, but much milder in anemia (often undetected) and without splenomegaly

Acquired disorders of RBC membrane structure that lead to hemolysis include alcoholic stomatocytosis (due to chronic abuse of alcohol) and hypophosphatemic stomatocytosis, both being correctable conditions. The latter is observed in alcohol withdrawal, diabetes mellitus and uremia, and reflects lack of dietary phosphate required for ATP formation and energy delivery to cytoskeleton proteins. The ATP lack affects a variety of key enzymes regulating the RBC membrane and cytoskeleton. Enzymes modulating phospholipid distribution (such as flippases and floppases, see accompanying communication [41]) will be turned off, and the activity of the cytoskeletal proteins that keep the RBC flexible will also shutdown. Thus, hypophosphatemic stomatocytosis has a profound analogy with hemolytic anemias caused by metabolic enzymopathies.

1.2.3. Hemolytic anemias caused by defective glycolytic metabolism (enzymopathies)

Persisting acquired hypophosphatemic anemia is wider in consequences than specific metabolic enzymopathies that also affect ATP generation. Also, these enzymopathies are genetic.

 ISSN 1920-3799

The autosomal recessive defect in the Embden-Meyerhof pathway that occurs only in homozygotes typically induces pyruvate kinase deficiency, and thus inability to convert glucose into lactate via anaerobic glycolysis. Since the glycolytic intermediaries accumulate owing to incomplete glycolysis, the levels of 2,3-diphosphoglycerate (DPG, which stabilizes deoxyhemoglobin and modulates the oxygen affinity of hemoglobin) are high and the affinity of Hb A for oxygen extremely low. With both energy metabolic circuits hindered (incomplete glycolysis and insufficient oxygen for respiration), the generated RBCs are energy deprived and lack ATP. This results in the production of crenated spherocytes (spherocytic echinocytes), indicating that deficient ATP-supply caused both spherocytosis and echinocytosis. Likely the former stemmed from non-contractility of the spectrin and actin cytoskeleton, and the latter was effectuated by the shutdown of flippases [41]. From our viewpoint, it is critical to realize the import of pyruvate kinase deficiency. It indicates that, at the limit, the combined effects of malnutrition and hypoxia result in both lack of energy to produce properly matured RBCs, and lack of energy to sustain the normal functioning of RBCs. Thus, RBC energy deprivation is translated into the combined morphological changes of, respectively, spherocytosis and echinocytosis. We will return to this subject in the accompanying paper.

The X-linked glucose-6-phosphate dehydrogenase defect is fully expressed in males and homozygous females, with variable expression in heterozygous females. It is best diagnosed by the presence of abnormally shaped RBCs that look like they have had one or more bites (bite cells). Hemolysis is precipiated by drugs (eg salicylates including aspirin, sulfonamides, nitrofurans, vitamin K derivatives, etc) and oxidizing agents (eg peroxide).

In hexokinase deficiency, the concentration of glycolytic intermediaries, such as DPG, is low owing to impaired glucose phosphorylation. Accordingly, in the absence of DPG, the uncapped HbA has an abnormally high affinity for oxygen.

1.2.4. Hemolytic anemias caused by defective hemoglobin synthesis (hemoglobinopathies)

Microcytic iron-utilization anemias already entail deficient hemoglobin synthesis, and thus encompass hemoglobinopathies of the thalassemic type, as well as sideroblastic or myeloblastic anemia that is now part of the myelodysplastic syndrome (MDS). These disorders involve defective erythropoiesis, but they also lead to hemolysis and thus promote the emergence of AIHA (see section 5 below on MDS). They belong, in fact, to hemolytic disorders caused by abnormalities of Hb. However, the term "hemoglobinopathy" is reserved to Hb abnormalities that are genetic, in the ordinary sense of familial or congenital. This definition encompasses the thalassemias but not sideroblastic anemia or myelodysplasia; the latter appear, in fact, to be acquired rather than hereditary disorders.

 ISSN 1920-3799

Hemoglobin is a complex, tetrameric, allosteric protein composed of two pairs of globularized globin polypeptide chains which, in the adult hemoglobin, Hb A_1, are designated as the alpha and beta chains. A small proportion (2.5%) of adult hemoglobin is also in the Hb A_2 form, made up by globin alpha and delta chains. In fetal hemoglobin, Hb F, the beta chains are replaced by gamma chains with a different amino acid sequence. Most importantly, in the presence of 2,3-diphosphoglycerate (DPG), Hb F has a higher oxygen affinity for oxygen than does Hb A. A normal adult may have up to 2% Hb F, but this proportion substantially increases in myeloid disorders to become a marker shared by aplastic anemia, generalized marrow aplasia, thalassemia major (see below) and myeloproliferative diseases (high Hb F is frequently found in PV, see below). This may well correlate with some degree of anaplastic reversion of myeloid cells or myeloblasts, but in our view it strongly suggests that the body's unconscious response to the hypoxia that accompanies these myeloid disorders is to take recourse to a hemoglobin form capable of optimizing oxygen transfer when DPG levels are normal.

One major group of hemoglobinopathies stem from amino acid substitutions in the beta chain, and encompass sickle cell anemia (SCA), Hb C disease and Hb E disease. The other major group comprises the thalassemias caused by a defect in the production of at least one polypeptide chain.

Discovered in 1904 by Dr. James Herrick, a Chicago physician, SCA requires homozygotes for its expression and is almost exclusive to black populations. It is caused by a substitution of valine for glutamic acid in the 6th amino acid of the globin beta chain. This is sufficient to decrease the negative charge of the globularized beta chain and trigger a characteristic shape change of the RBC to the form of a sickle. 'Sickling', as it is called, occurs over ectoplasmic or cortical zones of the RBC where the oxygen pressure is low and remains low. The molecular defect leads Hb to form a fibrous precipitate upon deoxygenation, and the precipitate deforms the red cells. The intensity of the deoxygenation or severity of RBC sickling correlates with the activity of superoxide dismutase (SOD) [42], suggesting that more than just an hemoglobinopathy may be involved in SCA. In homozygotes, half of the RBCs are sickle cells. Target cells are also frequent. The entire sickle cell is rigid, and has a tendency to interact with endothelial cells and plug capillaries and arterioles, forming an infarctus. The fragility of sickle cells leads to their hemolysis in circulation. In children hepatosplenomegaly is common, but in adults it is generally small because of infarctus-induced splenic fibrosis (a detectable spleen in an adult with sickle cells in peripheral blood is either a heterozygote or has hemoglobin of the S-C type). However, generalized adenopathy is common in adults. Reticulocytosis in peripheral blood is frequent, and so is leukocytosis. In fact, a generalized marrow hyperplasia is observed, including increased platelet production, and this may yield to aplasia upon severe infection.

 ISSN 1920-3799

Though somewhat contentious to this day, it is epidemiologically thought that the high incidence of the sickle gene in parts of Africa is evolutionarily due to the protection that it confers to heterozygotes against malaria [43-47], with the incidence of the latter correlating with the frequency of the sickle gene. Similar protection is afforded to Hb C heterozygotes and homozygotes [48]. The exact mechanism of the protection remains unknown to this day. It may be that the hypoxic environment of the sickle cell hinders the growth of the intracellular parasite [49], specifically the re-activation of its dormant mitochondria, but it has also been suggested that the sickling - provoked by invasion of the heterozygotic RBC with the malaria parasite - aggregates the membrane protein Band 3 (part of the ankyrin complex containing the Rh antigen) to promote increased binding of IgG to the Band 3 cluster, and thus to activate phagocytosis of the infected sickle cell by macrophages [50].

Hb E is the third most frequent hemoglobin (after Hb A and Hb S), and it carries a substitution of lysine for glutamic acid in the same 6th amino acid of the globin beta chain. The heterozygote (Hb AE) presents no peripheral abnormalities or anemia, but homozygotes (Hb E disease) exhibit a mild microcytic anemia with target cells. Hb C disease is also expressed only by homozygotes, but the anemia is normocytic, with target cells and spherocytes. If microcytes are also present and there is no iron deficiency, it is associated with alpha-thalassemia.

Alpha-thalassemia is due to decreased synthesis of the globin alpha chain, which is encoded by two pairs of structural genes. Heterozygotes for a double defect in each of the genes, or homozygotes for a defect in one of the two genes (double gene homozygosity is lethal), resort to the formation of tetramers of exclusively beta chains (Hb H) or, in infancy, of exclusively gamma chains. The microcytic anemia that results is mild to moderate, but it is accompanied by hemolysis and splenomegaly.

Beta-thalassemias are due to decreased synthesis of the globin beta chain. Heterozygotes have asymptomatic microcytic anemia (thalassemia minor), but homozygotes present severe anemia (thalassemia major or Cooley's anemia), splenomegaly, and hepatic siderosis leading to liver failure. The peripheral blood picture includes nucleated erythroblasts, reticulocytes, target cells. microcytic and hypochromic RBCs, with diffuse basophilia. As thalassemia major aggravates, Hb F may increase up to 90% of blood hemoglobin.

Note, then, that while a profusion of sickle cells in peripheral blood is diagnostic of SCA, all the other globinopathies present target cells, and these morphologically altered RBCs thereby become diagnostic of Hb disease or thalassemia.

1.2.5. Hemolytic anemias caused by mechanical injury

Traumatic or microangiopathic hemolytic anemia may be extravascular, due to injury or injurious practices (any form of a blow or an activity that may mechanically impact blood vessels and

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cells), or be vascular, typically caused by excessive shear, turbulence or thrombi. The result is fragmentation of RBCs, with anisocytosis (high RDW) and low MCV values, along with aberrant RBC shapes (triangles, echinocytes). It may or may not present splenomegaly. It should be remarked that the hemolysis caused by trauma is not circumscribed to the classical process of hemolysis that leaks hemoglobin and turns RBCs into ghosts. Her,e we have a clear example of RBC lysis by cytoplasmic fragmentation.

1.3. Anemias of infection

Anemias can also be caused by infection with prokaryotic or eukaryotic (protozoal) microorganisms. They generally hinder iron-homeostasis and are either normocytic or microcytic.

Amongst the anemias of bacterial infection, severe normocytic and often hypochromic anemia with intense hemolysis develops with tuberculosis, *Escherichia coli*, *Streptococcus haemolyticus* and *Clostridium welchii* infections, and in rheumatoid fever and subacute infective endocarditis associated with proliferation of a variety of bacteria (streptococci, staphylococci, especially *S. aureus*, and haemophilus species). The hemoglobin rarely falls below 9g/dL. In contrast with iron-deficiency anemias, the serum iron and total iron-binding capacity are both low. Typically, production of EPO and other cytokines is depressed and erythropoiesis is impaired. Most likely the impairment is not simply the result of depressed EPO and cytokine production, but involves other pathways affected by cytotoxins and iron metabolism.

Anemias caused by protozoal infections are just as severe as those caused by bacterial infections. The three main anemias of protozoal infection are caused by (1) parasites of the malaria genus *Plasmodium* (*falciparum* being the most common); (2) visceral leishmaniasis (Kala-Azar disease); and (3) trypanosomiasis (African sleeping sickness and American Chagas' disease).

Malarial diseases are still the leading cause of childhood mortality worldwide, with 90% of it occurring in Sub-Saharan Africa [51], with up to 3 million children under the age of five dying each year [52-54]. Malaria-causing plasmodium protozoa are apparently the only parasites that can infect human red blood cells, where they interfere directly with the function and the structure (morphology) of the host cell, and mature into a characteristic ring form. The parasite belongs to the protozoan phylum of the Sporozoa, whose cells have the ability to form spores (the sporozoites) that they employ in the passage from host to host, with both sexual and asexual life cycles. Malaria is transmitted in nature by the byte of the infected female *Anopheles* mosquito. The active stage (the gamont, either merozoite or trophozoite) emerges directly from the growth and asexual division of the sporozoite, through a process called "schizogony". The insidious process is initially exoerythrocytic, as it takes place inside an infected hepatocyte. Subsequently, the hepatocyte is ruptured and the merozoites enter the bloodstream to infect red blood cells (insidious endoerythrocytic phase) where they develop into

 ISSN 1920-3799

ring stage trophozoites [55]. The trophozoite catabolizes hemoglobin, using the globin chains as a source of amino acids [56]. Finally, the trophozoite develops into 8 to 24 daughter-cell merozoites which are released when the host RBC is ruptured. This release marks the beginning of the clinical disease presentation, mostly due to the dysregulation of the cytokine-mediated immune response of RE phagocytes. Some *Plasmodium* species (viz *P. vivax* or *P. ovale*) only induce benign disease, but others, such as the prevalent *P. falciparum* and *P. malariae*, are malignant. The normocytic anemia resulting from RBC infection is accompanied by severe poikilocytosis and marked anisocytosis. RBCs become rigid and sticky, so they tend to clump and originate infarctuses. Paroxystic crises present leukocytosis, but malaria evolves into a leukopenia and erythremia, with large, blast-like mononuclear cells, this apparently being the result of the protozoon's ability to infect myeloid stem cells. Splenomegaly, hemolytic jaundice and thrombocytopenia develop if the infection is severe. The mitochondria of plasmodium do not appear to function in energy generation the way they do in other eukaryotes, as they entirely lack the pyruvate dehydrogenase complex [57]. The discovery of an acetyl-Co-A generating plastid (termed an apicoplast) in plasmodium suggests that fatty acid supply is cytoplasmic and not mitochondrial. Thus the presence of an intact respiratory chain in the mitochondria of malaria parasites [58] seemingly indicates that the electron chain may be turned inside out (everted) in the mitochondria of the intraerythrocytic stages, even if it is normally oriented in motile stages (such as the sporozoite) [57]. Since treatment with quinine eliminates the endoerythrocytic stages, but not exoerythrocytic ones, the effect may well be connected with the different role of mitochondria in the two stages. Plasmodium is known for acquiring resistance to a variety of drugs, but not to quinine.

Leishmania from different species and subspecies cause visceral leishmaniasis, with progressive and marked splenomegaly and hepatomegaly, as well as progressive severe normocytic anemia and leukopenia, being distinctive symptoms in most cases. The genus Leishmania belongs, together with the genus Trypanosoma, to the protozoal phylum of the Mastigophora, order of the Trypanosomatida, and the same family of the Trypanosomatidae. Mastigophora are flagellated protozoa that may or not have chloroplasts. Whereas trypanosomes retain their flagellum in both the vertebrate and invertebrate hosts, leishmania do not in either case, only the leptomonad form of leishmania in the invertebrate host being flagellated. *Leishmania donovani* (cause of Kala-Azar) and *Leishmania brasiliensis* (cause of *Espundita*) are malignant diseases that eventually cause death. The parasite has two distinctive states. It is transmitted as the infective *promastigote* from the gut of the insect vector via the bite of the sandflies *Phlebotomus* and *Lutzomyia*. Its main strategy (a true Trojan horse) is to be phagocytosed by macrophages, inside of which it converts into the *amastigote* form which is able to grow and replicate in the phagolysosomes of macrophages. Leishmania resist killing by the high oxygen (typi-

 ISSN 1920-3799

cally hydroperoxides) and high nitrogen molecular species secreted into the phagolysosomes by the macrophages. The resistance appears to be due to the expression of peroxiredoxins by the mitochondria of the amastigote stage, which stabilize the membrane potential and prevent the mitochondria-induced cell death (apoptosis) of the parasite [59]. Normocytic-normochromic anemia accompanies the hypersplenism. The rapid splenomegaly is due to the monocytosis (usually absolute). In microscopically examined blood smears, the anomalous monocytes can be easily confused with the monocyte-like "starry-sky" large B cell lymphocytes of infectious mononucleosis (IM). The cells of the RE system respond to the parasitic infection by proliferating and providing therefore new hosts for infection by the parasite, while, simultaneously, becoming dysfunctional with respect to their normal role of clearing up defective RBCs, and of digesting and reprocessing their materials. The mononucleosis, together with the dysfunctional splenic clearance of RBCs, clog up the splenic sinuses. This is one of the factors contributing to the anemia. But, since the anemia is associated with both leukopenia and thrombocytopenia, it is likely that the monocytosis may not just exhaust a myeloid monocytic progenitor or stem cell, but also be the result of its infection by the parasite. This would be a second factor contributing to the anemia.

Anemia is a well-established immunopathological consequence of trypanosomiasis, and its intensity correlates with the severity of the infection. It is associated with splenomegaly in African trypanosomiasis (transmitted by the bites of tsetse *Glossina* flies), and with hepatomegaly in American trypanosomiasis (Chagas' Disease transmitted by the bites of reduviid bugs, often called 'kissing bugs' or 'cone-nosed bugs' from the genus *Triatoma*). In contrast to leishmania, the developmental stages of trypanosomes involve growth and proliferation of free forms in the gut, bloodstream and lymph nodes of vertebrates. Though they retain their flagellated state in both vertebrate and invertebrate stages, leishmanial (nonflagellated) forms are sometimes found in the invertebrate life cycle. The African disease is mostly due to *Trypanosoma gambiense* and *T. rhodesiense*, whereas Chagas' Disease is caused by *T. cruzi*. In the first phase of the disease, the anemia appears to be an anemia of chronic disease, typically microcytic, with an imbalance between the abnormal erythrophagocytosis in the spleen and the marrow erythropoietic response. The excessive erythrophagocytosis is driven by immune Type I cytokine-activation of macrophages (M1 cells), while the erythropoiesis is hampered by altered iron-recycling in macrophages [60]. Export of iron is accelerated by increased expression of genes for transferrin (Tf) and ferroportin-1 (FPN-1) [60]. In the second phase of the disease, the anemia becomes chronic and progressive, iron-export being hindered, and increased ferritin expression suggesting growing iron sequestration. Oxidative phosphorylation takes place in mitochondria of the promastigote or procyclic stage (in the midgut of the tsetse fly). But the slender, tubular mitochondria of trypanosome bloodstream forms lack a functional respiratory chain, as the trypanosome

 ISSN 1920-3799

Table 4
Diagnostic erythrocyte shape changes observed in the anemias

		1. Anisocytosis	2. Microcytes	3. Macrocytes	4. Megaloblastic macro-ovalocytes	5. Ovalocytes	6. Spherocytes	7. Sickle cells	8. Stomatocytes	9. Target cells	10. Byte cells	11. Poikilocytes	12. Echinocytes	13. Acanthocytes	14. Reticulocytes	15. Normoblasts	16. Sideroblasts	17. Hypochromia	18. Hyperchromia
Refractory	MICROCYTIC Fe deficiencies	+	+			+				+		+						(+)	
	Sideroblastic	+	+							+							+	+	
	NORMOCYTIC Aplastic	(+)		(+)											(+)			(+)	
	Myelophtisic											+			+			+	
	Myelodysplasia (MDS)	+			(+)							+				+	(+)	(+)	
	MACROCYTIC Nonmegaloblastic	+		+					+					+					
	Megaloblastic (Pernicious)	+		+	+							+							(+)
	Lack of Vit. C		(+)	(+)	(+)							+						(+)	
	HEMOLYTIC 1. AIA Phagocytic																		
	AIHAA						+												
	2. Membrane disorders MM syndrome																		
	HS						+								+				
	HE					+									+				
	Acquired (lack of PO_4)								+										
	3. Enzymopathies Pyruvate Kinase						+						+						
	GGPD deficiency										+			+					
	4. Defective Hb SCA							+							+				
	HbE disease	+	+							+									
	α-thalasseuria & β-thalassemria minor	+	+			(+)				+		+							
	β-thalassemria major	+	+			+				+		+			+			+	
	5. Trauma	+							+			+	+						

derives most of its energy from glycolysis at this stage. Still, the mitochondria manage to preserve a comparable membrane potential in bloodstream forms because of ongoing hydrolysis of ATP by ATP-synthase. In other words, ATP synthase is implicated in the maintenance of the mitochondrial potential in trypanosome bloodstream forms [61].

In light of the preceding - and with respect to anemias provoked by infections with Mastigophora - it is conceivable that any approach capable of cancelling the membrane potential of

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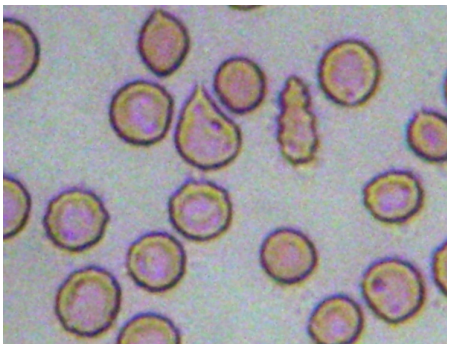
mitochondria (eg by preventing peroxiredoxin production by amastigotes, by reducing ATP synthase gene expression with RNA interference in bloodstream forms, etc) in parasites found at sensitive cell stages that rely upon glycolysis, might trigger mitochondria-mediated apoptosis of the parasite in vertebrate hosts. The approach would prevent trypanosomes from surviving in bloodstream and leishmania inside phagocytes, and thus preclude expression of the derived anemias.

It is evident that the anemias are many, and that most are merely symptoms and stages of much deeper disorders or disease vectors, mainly neoplastic or infectious, that affect erythropoiesis, hemolysis or both. Most erythropoietic anemias in fact lead to hemolysis. But it is also remarkable that hemolysis involves different processes - those associated with some form of auto-immunity (whether cellular or humoral), those associated with the classical process of hemolysis, and those involving the fragmentation of the cytoplasm of RBCs into cytoplasts. The abnormal and symptomatic shape changes of RBCs observed in all of the anemias are summarized for quick reference in **Table** 4. Examples of anisocytes, poikilocytes, echinocytes and acanthocytes are shown in **Fig.s 4A** and **4B**.



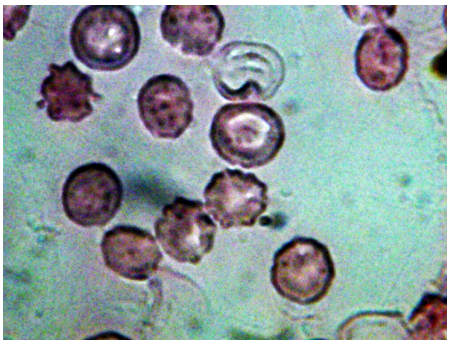


Fig. 4A (**left**) - Anisocytosis with poikilocytosis (abnormal forms including pear-shaped or tear-drop RBCs) in a peripheral blood smear. Wright's stain, daylight filter, Nikon Coolpix 5000, 1,100x print mag.
Fig. 4B (**right**) - Echinocytes (including one acantho-echinocyte), hemolyzed ghosts and a hemolyzing poikilocyte in a peripheral blood smear. Wright's stain, Blue monochromatic filter, Phase Contrast, Nikon Coolpix 5000, 1,100x print mag.

2. The stress, secondary and familial polycythemias

2.1. Stress and secondary polycythemias

Biological stress is recognized as playing a modulating role in the stimulation of erythropoiesis and erythroid progenitor proliferation. Stress, in this sense, refers to nonhypoxic factors - such as fatigue, fluid loss or infection - that may cause apparent excess of RBCs. Designated as "stress polycythemia", the condition is not a true polycythemic or 'erythrocytotic' disorder, because there is no

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real increase in the RBC mass, which remains normal - along with normal hematocrit, EPO production and arterial oxygen tension. When caused by loss of fluid from the blood, its characteristic symptom is decreased plasma volume - which artificially increases RBC density. This distinguishes stress polycythemia from the true polycythemias - all of which present a real increase in RBC density.

In contrast, secondary polycythemia is a true erythrocytosis presenting an excess of normal RBCs, but not necessarily a clinical condition. Only secondary polycythemia caused by EPO-producing renal tumors is a clinical disorder; it is also a chronic one. All other forms of secondary polycythemia have hypoxia as effective or proximal cause - whether caused by altitude, poor breathing, deficient hemoglobin, or exposure to excess carbon dioxide - as in tobacco smoking. Secondary polycythemia may be a transient response, as is the case with smokers, or a prolonged response, as in the effect of high-altitude. Hypoventilation syndromes, in particular functional ones caused by chronic inspiratory attitude (incomplete exhalation, diaphragmatic stasis, anoxic air retained in lower lung portions), can develop secondary polycythemia. The normal response to the resulting partial hypoxia is - as we have already discussed in a previous communication [1] - EPO production (by kidney mesangial cells). Primed (mediated and enhanced) by the insulin and insulinlike response circuits [62], hemin [62], retinoids [63] plus interleukin-3 (IL-3) [62, 64] and Stem Cell factor (SCF, Kit ligand, Steel factor) [62, 65-66], the Epo-mediated response increases both proliferation of BFU-E and CFU-E progenitor cells, and the recruitment of erythropoietic cells committed to differentiate and undergo maturation. As bone marrow stem and progenitor cells are stimulated to produce more RBCs, the response requires an adequate supply of iron. The effects of hypoxia and the associated IGF-I response are mediated by HIF-1 [67], which transcriptionally activates genes for glucose transport and glycolytic enzymes needed to support the marrow proliferative response that will result in expansion of the erythroid compartment.

2.2. Autosomal polycythemias

Autosomal dominant polycythemias are rare hereditary disorders characterized by high proliferation of erythroid progenitors (familial erythrocytosis), elevated RBC mass and hemoglobin concentration. They are frequently associated with hemoglobin mutants having a greater affinity of hemoglobin for oxygen [68]. Some instances have been shown to be associated with increased EPO production [69-70], and that has been the general assumption made for congenital erythrocytoses. In other cases, EPO receptor mutations have been observed which increased the EPO-sensitivity of erythroid progenitors or transfected cells [71-72], but the EPO-hypersensitivity is not observed in all cases [73], nor has it been confirmed by studies of cellular response in serum-free media. Moreover, other studies suggested that cases of familial and congenital polycythemia may not involve the EPO receptor at all [74].

 ISSN 1920-3799

Table 5
Differential for polycythemias

	Hct	RBC Masss	Plasma Vol.	Spleen	EPO production	Arterial O_2
Stress erythrocytosis	N	N	↓↓	N	N	N
Secondary polycythemia (hypoxia induced)	↑	↑	↓	N	↑	↓
Secondary polycythemia (renal tumor-induced)	↑	↑	N	N	↑	N
Polycythemia vera (PV)	↑	↑	N or ↑	Enlarged	↓	N to ↓

3. Polycythemia *rubra vera* (PV) and IGF-I hypersensitivity

3.1. What is Polycythemia *vera* (PV) and its diagnostic algorithm(s)

Polycythemia *rubra vera* (PV) is a chronic myeloproliferative disorder - and by all accounts it seems today to effectively be a neoplasm - first discovered in 1892 [75] and first identified as a new clinical entity in 1903 [76]. It is characterized by trilineal marrow hyperplasia, with the major emphasis placed in the erythroid lineage, in the form of an uncontrolled expansion of the erythroid compartment. The defect in control of the size of the erythroid cell population is instrinsic to the hyperplastic cells. Despite the increased red cell mass, the disease is cyanotic. As shown in **Table 5**, stress erythrocytosis, functional secondary polycythemia, malignant secondary polycythemia and polycythemia vera are distinguishable by their effects on RBC mass, plasma volume, spleen size or mass, EPO production and arterial oxygenation. Only PV presents splenomegaly associated with increased hematocrit and RBC mass while EPO production is often depressed. Arterial oxygenation is normal or moderately hypoxic in PV, and thus comparable to hypoxia-induced secondary polycythemia.

 ISSN 1920-3799

Clinical diagnosis of PV was, up until recently, based on the presence of 3 major criteria and 2 out of 4 minor criteria.

The major criteria of the algorithm were:

- increased total red cell mass (>32-36 mL/Kg)
- normal to slightly hypoxic oxygen saturation (typically >91%)
- enlarged spleen (in ca 50% of the cases)

The minor criteria were:

- increased WBC count in PB
- increased platelet count in PB
- increased neutrophil alkaline phosphatase in serum
- increased serum vitamin B_{12} or B_{12}-binding

Note that increased B_{12}-binding is bound to promote conditions propitious for pernicious anemia.

In the present communication, we shall retain the above criteria, as being far preferable to those suggested recently by the 2008 WHO reclassification of myeloid neoplasms [77]. The latter diagnoses PV by 2 major and 3 minor criteria. The economy of criteria is deceptive, as we shall see at length below.

The 2 major criteria of the WHO reclassification algorithm are:

- increased hemoglobin (>18.5 g/dL in men and >16.5g/dL in women)
- Presence of the *JAK2V617F* mutation (see below)

The 3 minor criteria are:

- bone marrow biopsy showing trilineal hypercellularity
- serum EPO level below normal
- endogenous colony formation in vitro

Whereas we do not see major objections to the replacement of the increased red cell mass criterion by that of excess hemoglobin, nor to the inclusion of the *JAK2V617F* mutation as one of the main criteria, it seems that the requirement for trilineal hypercellularity is rash and nonspecific (shared with other myeloproliferative neoplasms), and that the criteria for endogenous colony formation in vitro is *entirely spurious unless the culture assay is conducted in serum-free conditions*, *without EPO and with and without IGF-I* (see below). As we shall discuss ahead, the reader will see that we may well be better off with a different set of diagnostic criteria than those proposed by the 2008

 ISSN 1920-3799

WHO reclassification. Our proposal of a novel PV algorithm can thus be summarized as follows:

The major criteria for the diagnosis of PV should be 3, and in fact *they should suffice for the diagnosis*:

- increased total red cell mass (>32-36 mL/Kg) *or* increased hemoglobin (>18.5 g/dL in men and >16.5g/dL in women)
- Presence of the *JAK2V617F* mutation (nonspecific)
- IGF-I hypersensitivity in serum-free medium (PV-specific)

However, to be sure of the diagnosis, *either* 1 of the following 2 minor criteria should be included:

- endogenous colony or burst formation in serum-free medium, in the absence of EPO, and with and without IGF-I (see below)
- serum EPO level below normal

or, instead, 2 of the following 5 minor criteria:

- normal to slightly hypoxic oxygen saturation (typically >91%)
- splenomegaly
- bone marrow biopsy showing bilineal or trilineal hyperplasia
- increased platelet count in PB
- increased neutrophil alkaline phosphatase in serum

PV is a fatal condition presenting high red blood cell counts and hemoglobin for many years. It evolves (see **Table 6**) into a compensated myelofibrosis, followed by severe, sclerotizing myelofibrosis and refractory anemia [78], the so-called "spent phase" [79] - and sharing many of the character-

Table 6

Phase	Characteristics	RBC lifespan	Changes in RBC morphology
Early	1. ↑ intramedullary RBC production: erythroid hyperplasia 2. Fast ion plasma turnover 3. Increase in RBCs with HbF	Normal	When iron deficiency sets in • poikilocytosis • anisocytosis with microcytosis • polychromatophilia
Late	1. Ineffective intramedullary RBC production 2. Extramedullary myelopoiesis (metaplastic)	Decreased	During "spent phase:" • marked poikilocytosis • ovalocytes • elliptocytes • nucleated erythroblasts

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Table 7

Clinical stages of the Wasserman "natural history hypothesis" of PV

1. Asymptomatic phase
2. Erythrocytic phase
3. "Spent phase":
   myelofibrosis & myeloid metaplasia
4. Acute leukemia

istics observed with the normocytic refractory anemias of myelophtisis and MDS, including poikilocytosis. Thrombotic episodes often occur during the compensated phase. Involvement of deficient vitamin B_{12} supply will overlay pernicious anemia-like symptoms. 5% of all PV cases evolve into AML (see below), including acute erythroleukemia, but the incidence increases with age [80]. Progression to Philadelphia chromosome-positive (Ph+) CML has also been observed (see section 6 below). Median survival of PV patients is 13 years from onset of the polycythemia, and the mean age at diagnostic is 60 yr.

Wasserman first detailed the evolution of the disease with his "natural history hypothesis" (see **Table 7**) [81]. The manifestation of the disease is preceded by an asymptomatic phase. The first symptom is the erythrocytotic phase, followed by the "spent phase" that plunges the patient into an acute leukemia.

Several criticisms can be, and have been, addressed to Wasserman's theory of the clinical stages, and several corrections and alterations have been suggested from altogether different approaches. One can construct an improved "natural history" of PV as shown in **Table 8**. An improved clinical staging of the symptomatic phases will take into account that the disease begins with an erythroid hyperplasia that still seemingly permits normal RBC differentiation to take place. It progresses to marrow depletion, and thus produces refractory anemia (normocytic) [81-82]. The result of the depletion is marrow failure, first fibrotic and then sclerotic, aggravating the refractory anemia and often initiating a pernicious-type anemia. This forces the body to seek other sources of hemopoiesis, and extramedullary hemopoiesis with myeloid metaplastic characteristics appears in spleen, liver, lymph nodes and kidneys. Splenomegaly is most often the earliest manifestation of myeloid metaplasia. Eventually an acute myeloid neoplasia (AML) sets in, that further worsens the anemia(s). The short interval between PV and the manifestation of AML excludes the notion of prolonged survival in PV, and indicates that the vector of its pathology will not arrest at myelofibrosis or myeloid metaplasia.

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Table 8

Improved Staging of PV

CHRONIC PHASE	1. Marrow erythroid hyperplasia
	2. Marrow depletion (MDS-like) → refractory anemia
	3. Marrow failure myelofibrosis and sclerosis
	4. Extramedullary hemopoiesis: myeloid metaplasia
ACUTE PHASE	5. Acute myeloid leukemia: neoplasia
	6. Aggravation of anemia

3.2. The proximal etiology of PV: IGF-I hypersensitivity of BFU-E and CFU-GEMM

Normal erythropoiesis - and the normal response to transient secondary polycythemia - is driven in response to EPO stimulation of BFU-E and CFU-E progenitor cells. When EPO concentrations are low (as is the case with PV patients, since the erythrocytosis itself depresses EPO production), normal erythropoiesis switches to an IGF-I-dependent pathway that normally is ~100-fold less

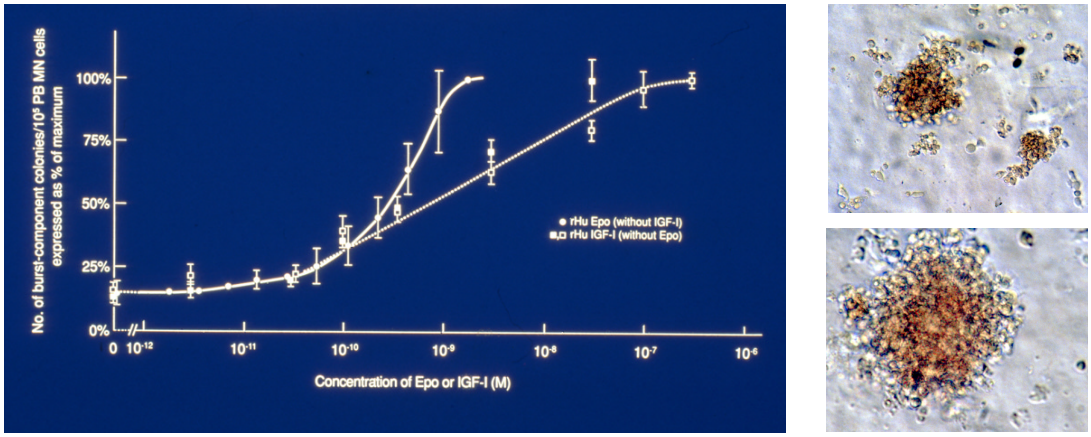

Fig. 5A - Normal erythropoiesis driven by either EPO or IGF-I. The graph presents a direct comparison of normalized values for d14 burst-component colony formation by circulating erythroid progenitor cells from normal subjects, as a function of rHu IGF-I or rHu EPO concentration in complete serum-free medium (after Correa & Axelrad [62, 131-132]).

Fig. 5B & C - Low (right top) and high (right bottom) power micrographs of live, fully hemoglobinized, EPO-independent, IGF-I-driven, normal erythroid bursts from PB MNC grown in completely defined serum-free medium. Respectively, 40x and 100x print magnifications. ASA64 Ektachrome daylight film.

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sensitive than the EPO pathway (see **Fig. 5A**) [83]. From in vitro studies in serum-free defined media, it became apparent that a small but significant percentage of the erythropoietic compartment is normally driven by IGF-I rather than EPO (normal EPO-independent bursts driven by IGF-I are shown in **Figures 5A & B**) [62]. Anti-EPO antibody only reduced the EPO-driven part of the compartment, and in the presence of IGF-I the same number of bursts was observed when the antibody was added as were driven by EPO in the absence of the antibody (see **Fig. 6**). Thus, under in vitro conditions, IGF-I is sufficient to drive erythroid differentiation of *normal* cells. This is a fundamental fact that

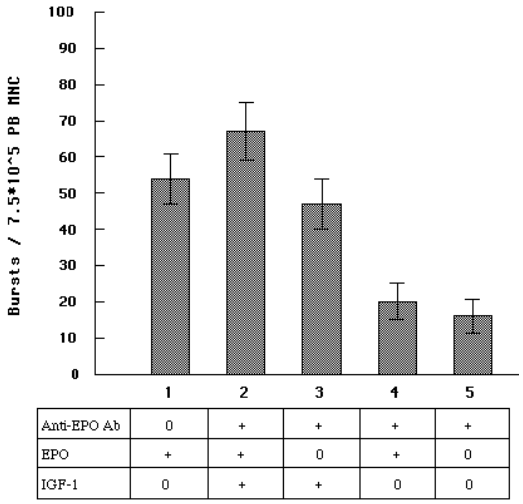

Fig. 6 - Effect of the addition of the polyclonal anti-EPO antibody HCC 1400 (1:50 dilution) to peripheral blood mononuclear cells (PB MNC) grown in a true serum-free medium with different combinations of rHu EPO (6 U/ml) and rHu IGF-I (300 µmol/L). At the dilution employed, HCC-1400 neutralizes 6 U/mL of EPO. (After Correa & Axelrad [87]).

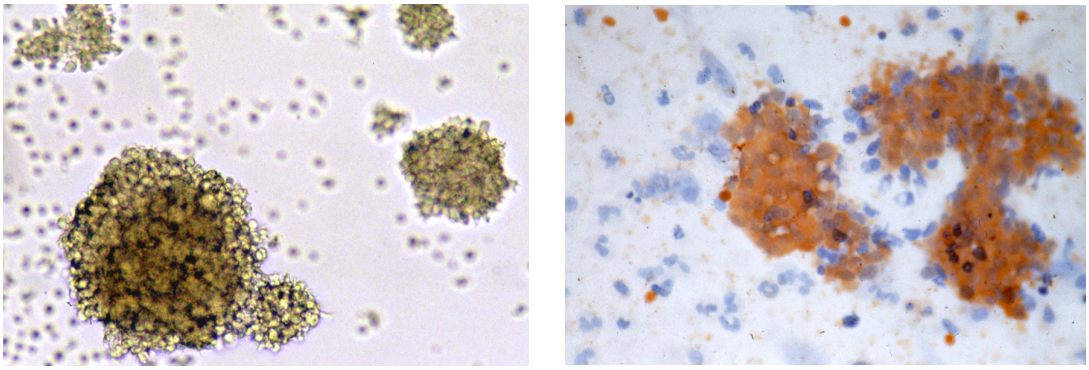

Fig. 7 - EECs, or endogenous erythroid colonies or bursts from PV PBMNC or PV marrow grown in 10% fetal calf serum without EPO. The colonies and bursts hemoglobinize poorly and must be stained to score for hemoglobinized cells. **Fig. 7A (left)** - Unstained, live d9 EEC's from PB MNC in the presence of added hemin. Despite addition of hemin, the hemoglobinization is weak. 100x print mag. **Fig 7B (right)** - Stained d9 EEC's with Benzidine-Haematoxylin. 100x print mag,

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continues to be largely ignored, and the IGF-I role in erythropoiesis is often wrongly reduced to a supportive one - as exemplified by a recent 8-author study [84] which reinvented the wheel of serum-free media while managing to entirely ignore the 2-decade-old findings of Correa and Axelrad regarding the IGF-I-driven complete maturation and growth of CFU-GEMM and BFU-E from peripheral blood in truly serum-free media.

One of the fundamental diagnostic observations of PV was the discovery that mononuclear cells (MNC) from the peripheral blood (PB) of patients affected with the disorder could give rise to erythroid colonies and bursts *in serum-containing cultures* performed without added EPO (see **Fig.** 7) [85-86]. This phenomenon became known as endogenous (or spontaneous) erythroid colony (EEC) or burst (EEB) formation, and led to the hypothesis that PV erythroid progenitors were either independent from EPO or hypersensitive to minute quantities of it present in serum [87].

Evidently, the erythroid colonies and bursts that Correa and Axelrad obtained by growth of normal PB MNC in serum-free cultures (**Fig.s 5A &B**) containing no EPO but added with IGF-I, mimicked the EECs and EEBs obtained by growth of PV PB MNC in serum-containing cultures with no EPO (**Fig.** 7). Thus, Correa and Axelrad decided to investigate the erythropoietic IGF-I response of PV progenitor cells, since it could well hold the key to understand the EEC phenomenon characteristic of PV. We should note that, unlike the EPO-pathway, the IGF-I pathway is hypoxia-independent. Correa and Axelrad found that in PV, the sensitivity of erythroid progenitors to IGF-I was increased more than 100-fold (see **Fig. 8**). Consequently, the IGF-I response of PV erythroid progenitor cells in the absence of EPO becomes comparable to the normal sensitivity of EPO (as shown in **Fig. 5A**), and is capable of explaining the production of endogenous erythroid colonies and bursts in serum-containing cultures of PV progenitors, *since serum is known to contain IGF-I* [88]. Simplified assays that even recently have been proposed as standards [89], typically contain high serum (eg 30%) and cannot, therefore, serve as as true EEC/EEB assays of PV, nor can they discriminate between different types of polycythemias, or eliminate background growth by normal erythroid progenitors.

In serum-free medium conditions and in the absence of EPO, it suffices to add IGF-I to obtain fully hemoglobinized burst-component colonies (BCCs) and bursts from PV PBMNC. **Fig. 9A** shows 2 bursts in serum-free medium, with one (on the right side of the plate) being composed by 3 BCCs. Similar bursts with 3 BCCs each from two other PV donors are shown in **Fig.s 9B** and **9C**. **Fig. 10A** shows a high power view of an EPO-independent single colony burst, and **Fig 10B** shows a very high power power view of the central BCC of a burst with 3 visible component colonies. For purposes of comparison to these EPO-independent, IGF-I-driven PV bursts, bursts from PV PBMNC grown in serum-free medium with EPO alone (in the absence of IGF-I), or with EPO and

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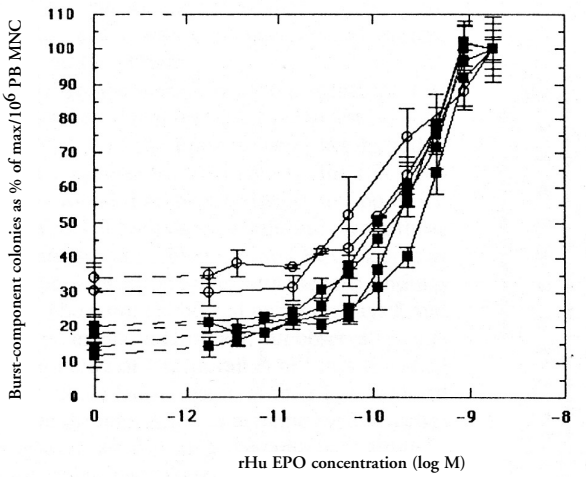

Fig. 8A - Comparison of normal vs PV BCC (burst-component colony) formation by circulating erythroid progenitor cells grown in serum-free medium, as a function of rHu EPO concentration, in the absence of IGF-I and expressed as a percentage of maximal rHu EPO stimulation (after Fig. 8 of Correa & Axelrad [88]).

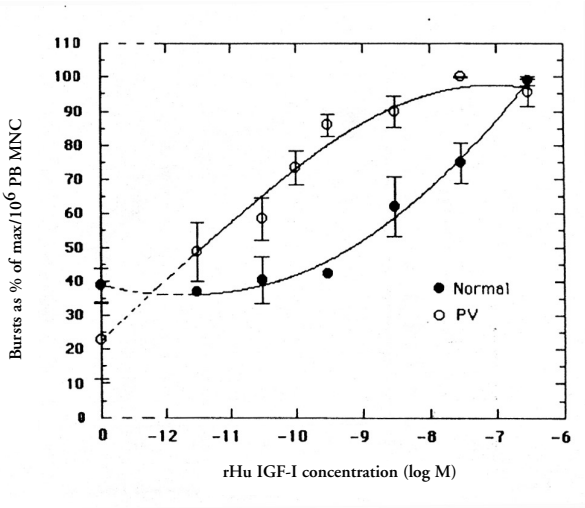

Fig. 8B - Comparison of normal vs PV burst formation by circulating erythroid progenitor cells grown in serum-free medium, as function of rHu IGF-I concentration, in the absence of EPO and expressed as a percentage of maximal IFG-I stimulation. PV patients: n = 5. Normal donors: n = 3. (After Fig. 9 of Correa & Axelrad [88].)

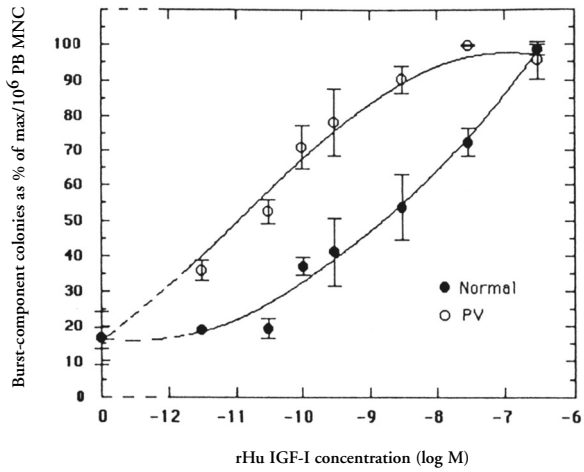

Fig. 8C - Comparison of normal vs PV BCC formation by circulating erythroid progenitor cells grown in serum-free medium, as a function of rHu IGF-I concentration, in the absence of EPO and expressed as a percentage of maximal IFG-I stimulation. PV patients: n = 5. Normal donors: n = 4. (After Fig. 10 of Correa & Axelrad [88].)

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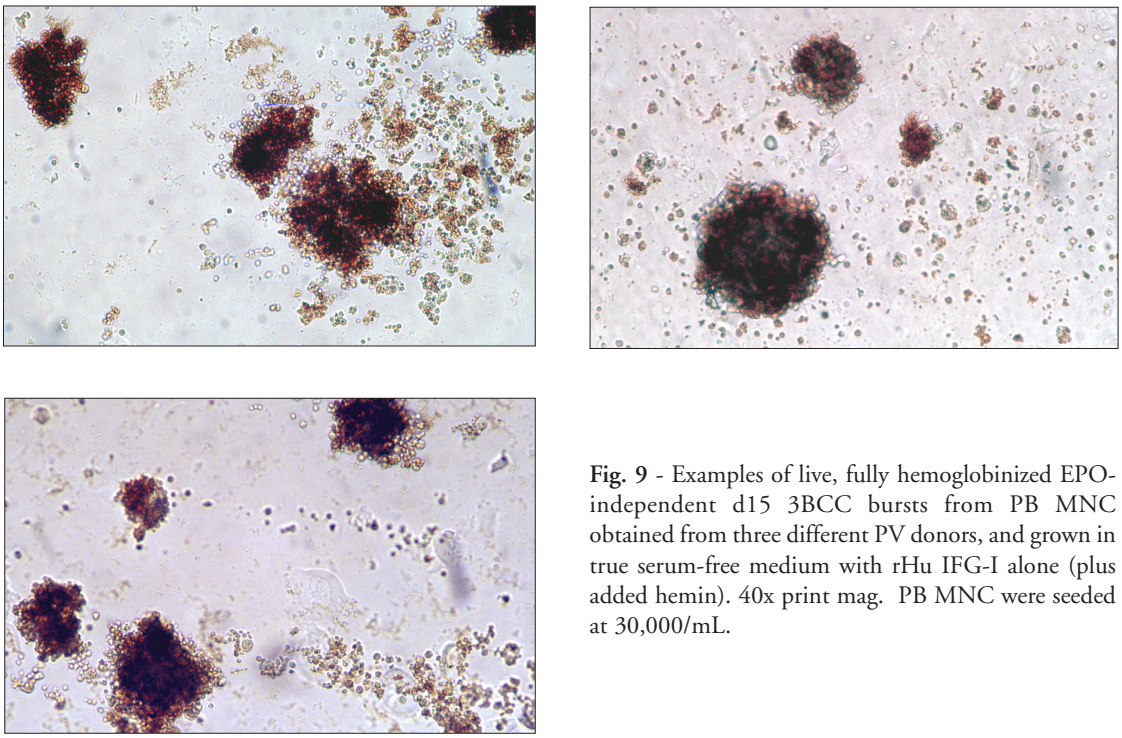

Fig. 9 - Examples of live, fully hemoglobinized EPO-independent d15 3BCC bursts from PB MNC obtained from three different PV donors, and grown in true serum-free medium with rHu IFG-I alone (plus added hemin). 40x print mag. PB MNC were seeded at 30,000/mL.

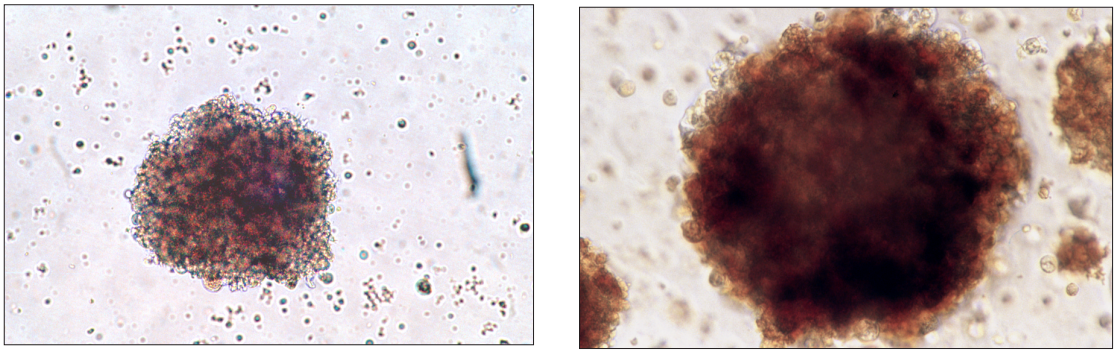

Fig. 10A (left) - High power view of a live EPO independent, IGF-I hypersensitive single-colony burst from PV PB MNC, grown in true serum-free medium with rHu IGF-I alone (plus added hemin). 60x print mag.
Fig. 10B (right) - Very high power view of a live EPO-independent, IFG-I hypersensitive, 3BCC-burst from PV PBMNC, grown in true serum-free medium with rHu IGF-I alone (plus added hemin). 125x print mag.

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IGF-I, are shown, respectively, in **Fig.s 11A & 11B**. It is evident that the EPO response of circulating erythroid progenitors in PV is normal, despite the differentiation by anti-EPO receptor monoclonal antibodies of EPO-dependent and EPO-independent BFU-E populations in the marrow and PB of PV patients [90]. The obvious suggestion is that once committed to an EPO-independent pathway, the BFU-E no longer need to express either the EPO receptor or be inhibited in their differentiation if the EPO-R is blocked.

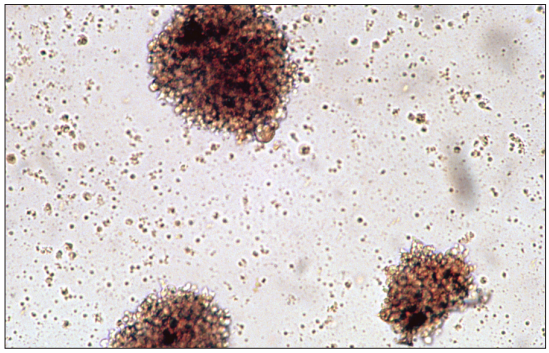

Fig. 11A - Fully hemoglobinized EPO-dependent d15 3BCC-burst from PV PB MNC grown in true serum-free medium in the absence of IGF-I and with rHu EPO alone (plus hemin). 90x print mag.

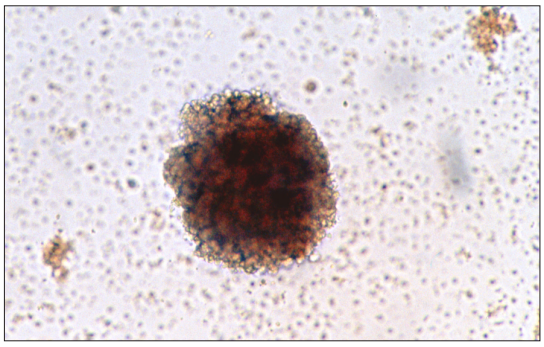

Fig. 11B - High power view of a single colony d15 burst from PV PB MNC grown in true serum-free medium, with rHu EPO, rHu IGF-I, hemin and rHu IL-3. 90x print mag.

Correa & Axelrad also formally demonstrated that the IGF-I-hypersensitivity of circulating erythroid progenitors in PV patients is mediated by the IGF-I receptor (IGF-IR), and anti-IGF-IR antibody eliminated 65-70% of the EPO-independent burst (BFU-E) and burst-component (BCC) colonies in PV (see **Fig.s 12A & 12B**). Damen et al confirmed the hypersensitivity results, when they found that cells expressing truncated EPO receptors were hyposensitive to EPO in the absence of serum, and their hyperproliferative response was actually hypersensitive to IGF-I and depended on the presence of IGF-I in serum [91].

Since the studies of Correa, Axelrad and their co-workers were carried out with serum-free defined media, the IGF-I-independent *and* EPO-independent bursts and BCC's could be formally

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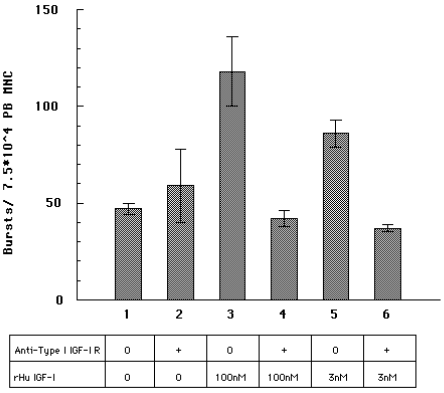
shown to be the result of the addition of hemin. Even though either retinyl acetate (RA, vitamin A) or all-*trans*-retinoic acid (ATRA) were added to the serum-free cultures to complete the medium required by normal erythroid progenitors, PV erythroid progenitors did not require retinoids in the presence of IGF-I, and the addition of retinoids had no detectable effect (see **Fig 13**). Thus, aside from EPO-independence, PV progenitors are also retinoid-independent. We should remark, in this



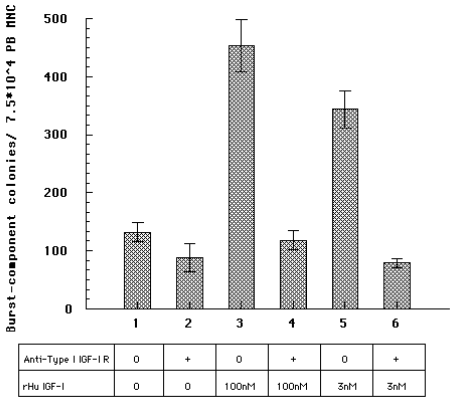


Fig. 12A (left) - Effect of anti-Type I IGF-IR antibody upon d15 erythroid burst formation by PV PB MNC in true serum-free medium and in the absence of EPO. All cultures contained 0.8nM rHu IL-3 and 0.25 mM hemin. (After Correa & Axelrad [88].)

Fig. 12B (right) - Corresponding effect of the same IGF-IR antibody upon d15 erythroid burst-component colony formation. (After Correa [83].)

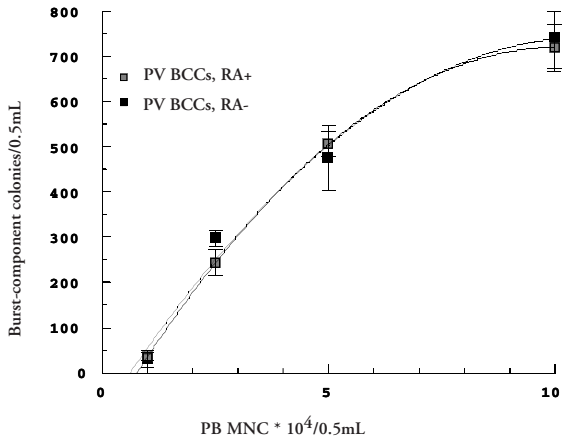


Fig. 13 - Effect of retinyl acetate (Vitamin A acetate) on true serum-free medium burst-component colony formation from PV PB MNC, at 10,000 to 100,000 cfu/mL. All cultures contained 0.8nM rHu IL-3, 0.25 mM hemin and 30nM rHu IGF-I.

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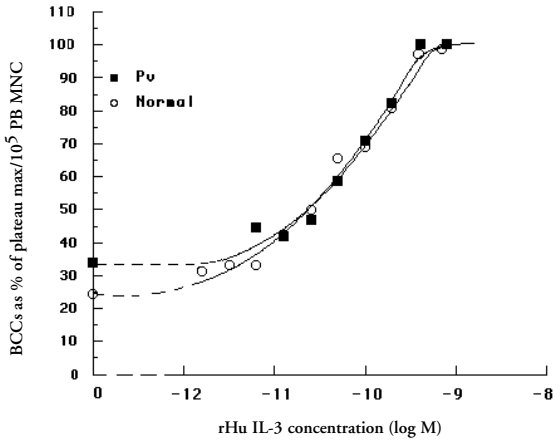

Fig. 14 - Comparison of typical burst-component colony formation by PV and normal PBMNC in true serum-free medium containing rHu EPO and rHu IGF-I, as a function of rHu IL-3 concentration. (After Correa [83].)

context, that a parallel *background* erythroid colony and burst formation could be observed in serum-free medium with *normal* PB MNC, when just hemin *with* retinoids were added [63]. However, in the absence of all cytokines (including retinoids) and hemin, normal PB MNC failed to generate erythroid colonies and bursts. Lastly, as exemplified by **Fig 14**, there was no difference in sensitivity found for the response of normal or PV PB MNC to rHu IL-3 in serum-free medium, in the presence, respectively, of plateau concentrations of both IGF-I and EPO.

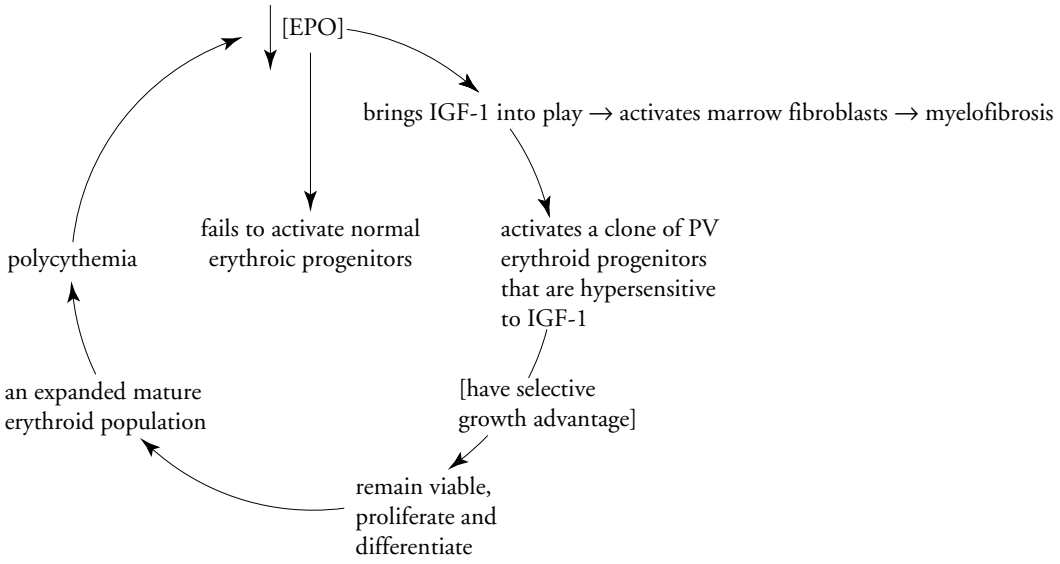

Fig. 15 - The Correa & Axelrad model of growth factor influences in Polycythemia vera. (After Correa [83] and Axelrad et al [92,97].

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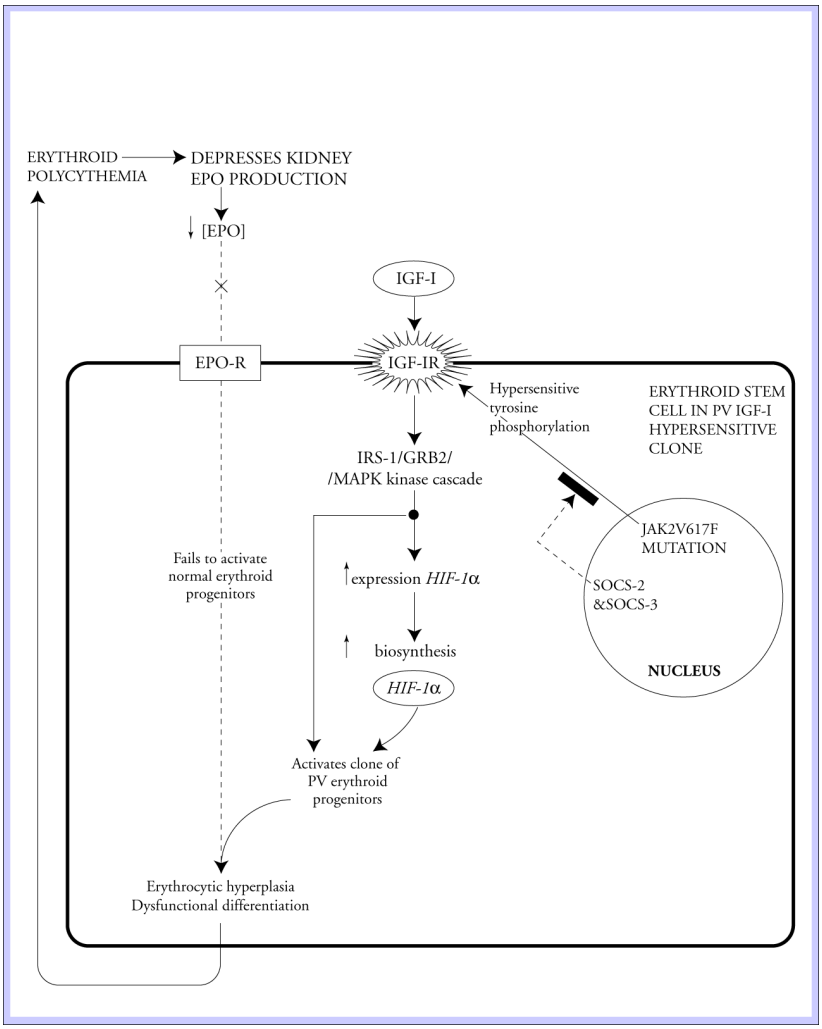

Fig. 16 - The Correa & Axelrad model of IGF-I hypersensitivity in PV erythroid progenitor cells (After figure 19 of Correa & Correa [1].)

These findings led to a model of erythropoietic regulation (the Correa&Axelrad model [83, 92]), shown in **Fig.s 15 & 16**, where the decreased EPO concentration triggers the recursive IGF-I circuit, and this, in turn, triggers a clone of PV erythroid precursors (BFU-E and likely CFU-GEmM) that are hypersensitive to IGF-I, and thus have a growth advantage. Simultaneously, IGF-I activates marrow fibroblasts, promoting myelofibrosis and anemia (the link to MDS) once the erythrocytosis will exhaust the marrow. The PV clone expands to produce the erythrocytosis characteristic of PV, which, in turn, further depresses EPO production by kidney mesangial cells. IGF-I receptors are expressed in normal RBCs, and both BFU-E and CFU-E respond to the ligand.

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The main adherent stromal cell population involved in controlling the IGF-I axis is monocytic, and it fundamentally responds to Growth Hormone (hGH):

hGH —> Monocytes —> IGF-I —> Erythroid progenitor growth (BFU-E, CFU-E)

The importance of the Correa & Axelrad discovery of IGF-I hypersensitivity as the biological basis of the erythroid-specific phenotype of PV is that it shed new light on the critical involvement of the GH/IGF-I axis in the regulation of erythropoiesis, and specifically on EPO-independent RBC production. This causal relation between GH or IGF-I and the development of PV is underlined by the observation of PV remission upon treatment of acromegaly [93].

However, the fact that PV often coexists with granulocytic and megakaryocytic hyperplasias underlines the ultimately pan-myeloid nature of the disorder. Increased RBC production in PV is not due to increased recruitment of BFU-E by a switching of a pluripotent stem cell progeny down the erythroid pathway at the expense of other myeloid lineages [94], but the EPO-independent phenotype associated with the disease is expressed at the BFU-E stage [95]. It is therefore likely that other defects in the myeloid stem cell compartment, in particular at the level of the CFU-GEMM, are involved in the etiology of PV. One such defect is the acquired *JAK2V617F* mutation (see below), but this is not specific to PV. A recent micro-array study of 9 patients has identified altered regulation of 11 genes in PB CD34+ cells [96], but it is doubtful whether any of these alterations are specific or even germane to PV.

Mixed myeloid d21 CFU-GEMM colonies obtained from normal PB MNC (see **Fig.s 17A & B**) or normal marrow MNC (see **Fig.s 18A & 18B**, respectively without and with rHu SCF) can best be grown in true serum-free media (in practice defined by zero background growth [62]) in the presence of the complete (and serum contaminant-free) cytokine complement (hemin, retinoids, rHu EPO, rHu IGF-I, rHu IL-3) plus rHu-SCF (stem cell growth factor) [66]. The spectacular multi-lin-

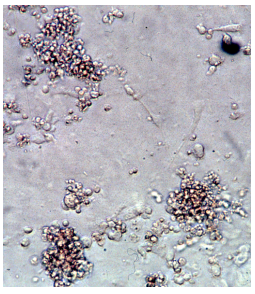

Fig. 17A (left) - Live, partially hemoglobinized, non-adherent cell-depleted d19 CFU-GEMM pan-myeloid colony cluster from normal PB MNC grown in serum-free medium with hemin, retinoids, rHu EPO, rHu IGF-I, rHu IL-3, but without SCF. 20x print mag.

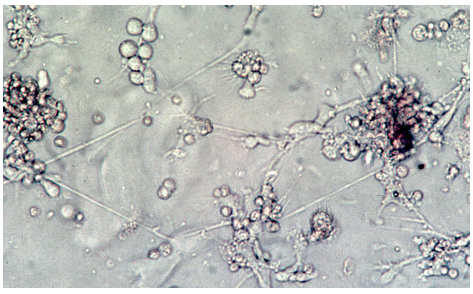

Fig. 17B (right) - Note the spontaneous reconstitution of a hematon-like structure, with erythroid colonies and megakaryocyte and granulocyte progenitors attached to the same network of stromal cells 125x print mag.

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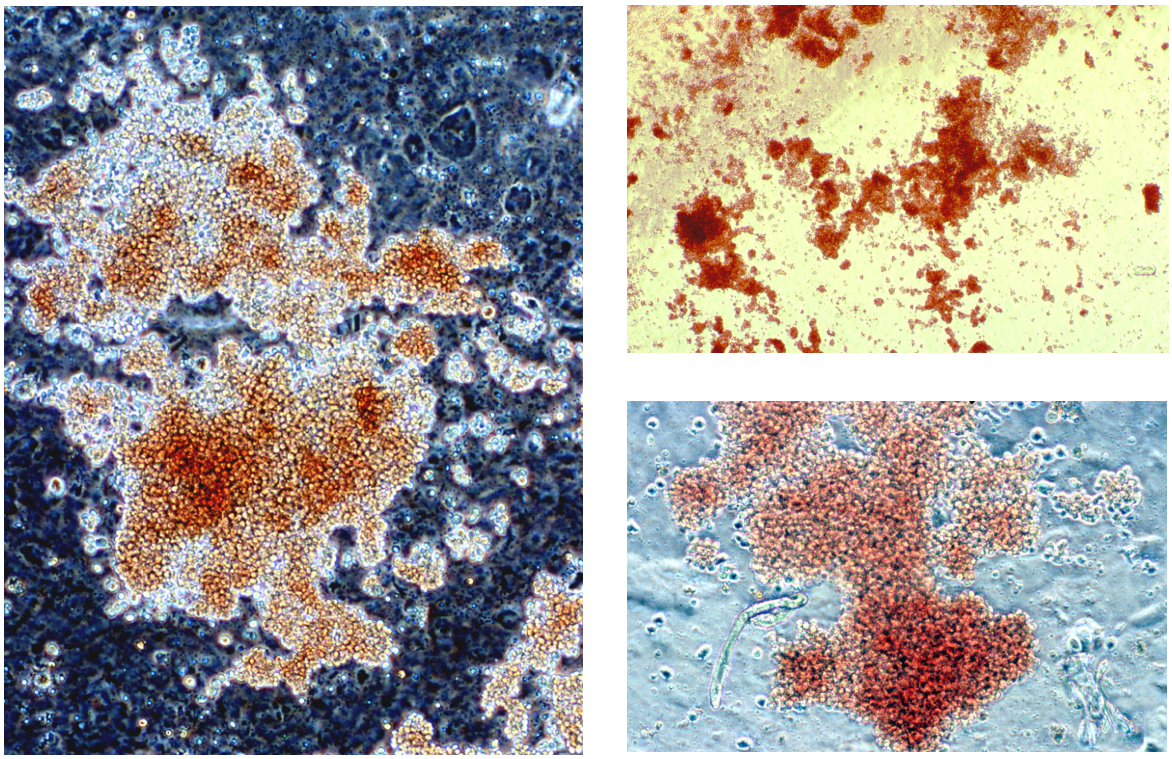

Fig. 18A (left) - Normal live d21 CFU-GEMM pan-myeloid colonies from marrow MNC grown in serum-free medium with the full complement of cytokines, in the absence of added SCF. Erythroid colonies are recognizable as distinct foci of hemoglobinization surrounded by nearly contiguous myeloid (granulocytic, monocytic and megakaryocytic) growth.Phase contrast invertoscope with monochromatic blue filter, ASA64 Ektachrome film, 60x print mag.
Fig. 18B (upper right) - Low power field of view of normal live d21 CFU-GEMM fully hemoglobinized pan-myeloid-colony from marrow MNC grown in serum-free medium with the full complement of cytokines plus added rHu SCF. The erythroid islands are practically contiguous in a mega pan-myeloid colony. Brightfield, ASA64 Ektachrome film, 10x print mag.
Fig. 18C (lower right) - High power view of normal live d21 CFU-GEmM pan-myeloid colony from marrow MNC grown in serum-free medium with rHu SCF and the full complement of cytokines except for addition of rHu IL-3. Daylight filter, ASA64 Ektachrome film, 65x print mag.

eage myeloid growth obtained in true serum-free culture at low seeding cell concentrations with the complete cytokine complement plus SCF (shown in **Fig 18B**) recapitulates under in vitro conditions the entire marrow CFU-GEMM-derived hemopoiesis (cp with **Fig. 1**). The proliferative stimulus of IL-3 can largely be replaced by addition of SCF (see **Fig. 18C**). Control of myeloid development along each lineage is cytokine-mediated, and can thus be pushed in serum-free culture towards megakaryopoiesis by addition of TPO, and towards granulopoiesis and monopoiesis by the addition of GM-CSF. Correa & Axelrad also showed that the effect that adherent cells in PB MNC preparations have upon burst formation, could be restored by addition of rHu SCF when adherent cells were

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depleted [66], and thus that presence of these cells in serum-free culture was sufficient to provide an undefined SCF quantity. In contrast, in PV, d21 CFU-GEMM growth, whether from PB or marrow, appears to be independent from the addition of SCF, and the presence or absence of adherent cells, or adherent cell factors [97-98]. Even though the mean concentration of CD34+ cells in PB MNC, but not in bone marrow, is significantly increased in PV patients compared to healthy controls [99], the CFU-GEMM progenitors from PB MNC of PV patients fail to respond to SCF in serum-free culture, when Il-3 and IGF-I are present [98]. It would seem therefore that either expression of CD34+ is not correlated with CD117(the SCF receptor) expression in PV (unlike what happens in AML, see below), or that most CD117 expressed in CD34+ cells from the PBMNC of PV patients have already been exposed in vivo to its ligand SCF. These findings underline what Prchal and Prchal wrote a decade and a half back, on the methodology that we employed:

"Correa and Axelrad developed a truly serum free system for the culture of erythroid [and myeloid] progenitors that removes the unpredictable variations associated with the use of uncharacterized biologic materials containing containing known and unknown [hematopoietic] activators and inhibitors." [100]

Zsebo and al [65] had already run into this problem when investigating the normal effect of SCF - for its effect appears to be greater when added to serum-contaminated media, than when added to true serum-free media (see **Fig. 18**) [66].

Cell lines derived from myeloblasts found in the PB of PV patients can also give rise to CFU-GEMM colonies in serum-free medium without addition of SCF (unpublished results). Some of these PV myeloblasts and erythroblasts present intense natural blue fluorescence of their nuclei (see **Fig.s 19A & 19B**) and sometimes of their mitochondria (**Fig. 19B**), and an intensely basophilic cytoplasm that is often vacuolated (see **Fig. 20**). Normal blast cells from bone marrow show no intense natural fluorescence. In some cases, the isolated PV cell lines included a B cell lymphoid component in their blast cell population, since the blasts consistently typed CD19+ (a B cell precursor and an early pre-B cell marker, whose expression is controlled by CD22 and its soluble variants), suggesting that some of the vacuolated blasts were "starry-sky" large B cell lymphocytes. We did not check whether these cell lines were unwittingly infected with EBV - an unlikely event, given that these lines were obtained from six different donors, given the sterile procedures involved in serum-free tissue culture and the fact that our lab never conducted research with EBV-producing cells. Moreover, B cell lineages have been previously observed in murine CFU-GEMM cultures [101], and there is evidence indicating that some of the circulating B cells in PV differentiate from the stem cell clone responsible for the disease [102].

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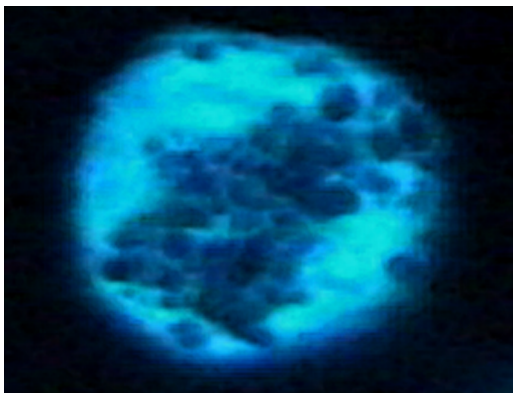

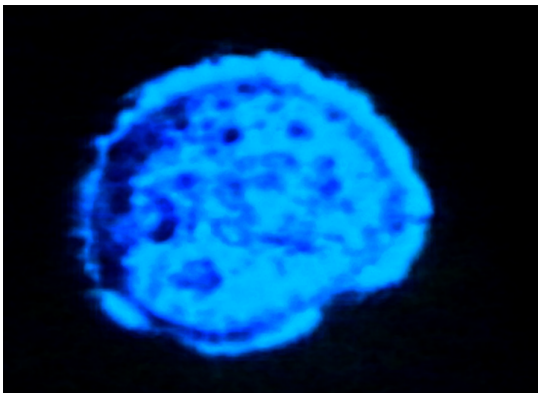

Fig. 19A (**left**) - Natural blue fluorescence of the nucleus of a PV myeloblast. Note the profusion of supranuclear organelles. Epifluorescence condenser. 2,800x print mag.
Fig. 19B (**right**) - Natural fluorescence of the nucleus, cytoplasm and supranublear organelles of a PV myeloblast. Epifluorescence condenser. 2,600x print mag.

When these findings - of an IGF-I hypersensitivity coupled to independence from factors such as EPO, retinoids and SCF - are taken together with the Correa & Axelrad model of erythropoiesis, it becomes possible to explain how, in PV, normal erythroid progenitors are suppressed in favor of aberrant ones, and independently of hypoxia. The erythrocytosis characteristic of PV is driven by an hypersensitivity mediated by IGF-I. Quoting from a review by Heike Pahl:

"Since the initial report by Prchal and Axelrad, a long debate has ensued as to whether PV cells are hypersensitive to or in fact independent of EPO. Because most colony assays were carried out in the presence of serum, it was feared that minimal quantities of EPO could be present in these assays and that these were sufficient to stimulate the hypersensitive PV cells. However, the addition of anti-EPO or anti-(EPO receptor) Ig did not abolish EEC formation in PV patients. The controversy was resolved by Correa et al in experiments using a novel serum-free medium. These researchers conclusively demonstrated that PV erythroid progenitor cells are independent of EPO. These experiments revealed that PV cells are hypersensitive to IGF-I." [103]

The IGF-I hypersensitivity is mediated by increased tyrosine-phosphorylation of the IGF-I receptor (see **Fig. 21**) [104]. Our findings identified two distinctive molecular features of the PV phenotype: (1) an increased *basal* tyrosine phosphorylation of the IGF-IR beta subunit, and (2) a substantially increased level of *induced* tyrosine phosphorylation of the receptor at lower concentrations of IGF-I, occurring earlier and attaining a higher level than in cells of healthy individuals or patients with secondary erythrocytosis [104]. We also found that the IGF-I hypersensitivity was not translated into an increase in the number of IGF-I receptors in PV [104], and that the amino acid sequence of

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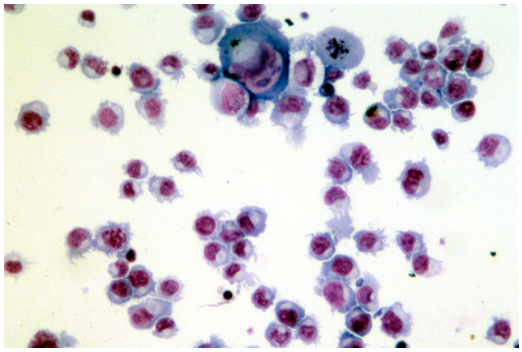

Fig. 20 - Smear of PV myeloblastic cell line grown in liquid serum-free medium (Wright-Giemsa stain). 200x print mag.

the IGF-I receptor is neither mutated nor deleted [92].

Receptor tyrosine kinases are critical enzymes that mediate the effect of extracellular growth factors on cell proliferation, metabolism and differentiation. Consistent with this view, an abnormal increase in the activity of receptor tyrosine kinases has been associated with unregulated growth (hyperplasia) and increased rates of glycolysis (the Warburg effect [1]). The insulin and insulinlike receptors have been implicated in the modulation and regulation of both proliferation and metabolism, as part of a major axis controlling the cell cycle (see **Fig. 21** and the detailed presentation in [1], whose figure 15 is here reproduced as **Fig. 22**). The IGF-IR has intrinsic tyrosine kinase activity [105], and normally functions during erythropoiesis to control cellular growth, differentiation, and inhibition of apoptosis [106-108]. The IGF-IR is known to act as an anti-apoptotic factor via the MAPK pathway (the kinase cascade, see **Fig. 22**) that controls both metabolism and growth [109]. The IGF-IR directly phosphorylates the insulin receptor substrate 1 (IRS-1) which interacts with the GRB-2 and Ras proteins to also activate the MAPK pathway. Just as importantly, the IGF-IR functions during neoplastic transformation [107]. These various functions of the IGF-IR have been mapped to different domains of the receptor [107], and have been shown to be mediated through different recursive pathways [110].

More recent work has shown that the majority of PV patients present a unique somatic mutation (*V617F*) that leads to the constitutive expression of JAK-2. This would constitute the initial molecular defect in PV. The mutation renders target cells hypersensitive to IGF-I - thus confirming the findings of Correa and Axelrad - and activates JAK1, Tyk2, and STAT3 and STAT5 constitutively (see **Fig. 22**) [111]. Hence a molecular model now exists that consistently explains both (1) the observed IGF-I hypersensitivity of PV erythroid progenitor cells (CFU-GEMM, BFU-E, CFU-E) in defined serum-free culture-media and in the absence of exogenously added EPO, and (2) the production of EEB/EEC in serum-containing tissue-culture contaminated with insulin or insulin-like factors, or by prior exposure to serum or cytokine secretions by adherent stromal cells.

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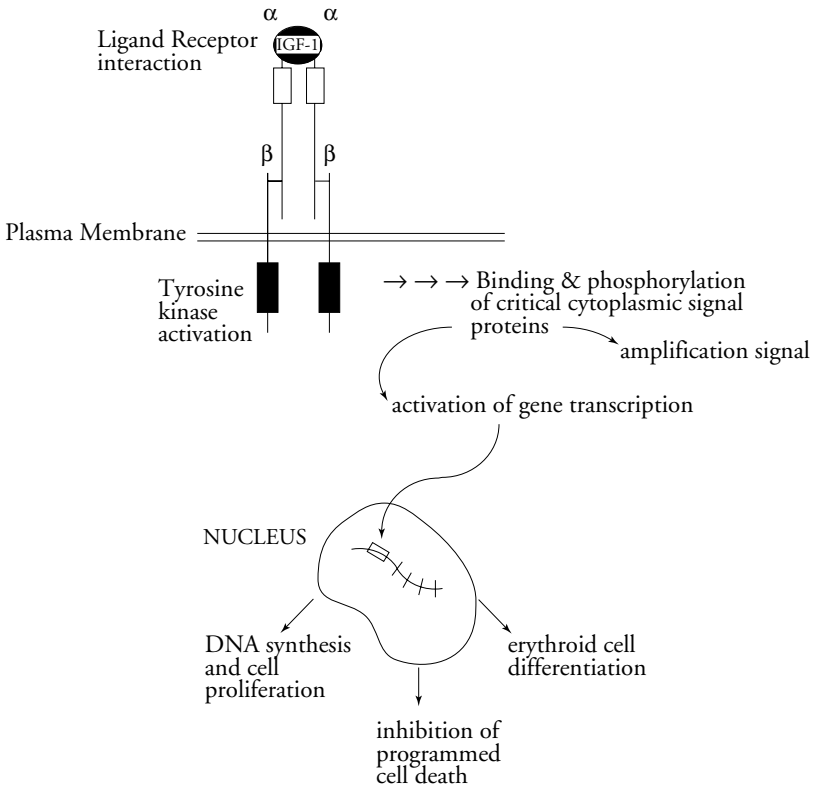

Fig. 21 - Schematized IGF-I receptor signal transduction pathways.

Recently published further investigation by our group revealed that suppressors of cytokine activity - encoded by *SOCS-2* and *SOCS-3* genes [112] (see below) - play a fundamental role in modulating the hypersensitivity response to IGF-I [113]. Overexpression of these genes is able to reverse both the IGF-I hypersensitivity and the PV erythrocytosis [113]. The observed increase in expression of *SOCS-2* and *SOCS-3* genes under the influence of IGF-I in normals and the reduction in expression of these genes in PV could be explained on the basis of suppression of cytokine signaling in the former, and of a defect in this suppressive function in the latter. In other words, the PV phenotype would be explained by two cardinal features: reduction of *SOCS-2* and *SOCS-3* expression, which would promote an increase in the number of erythroid progenitor cells (overgrowth); and reduced requirement for ligand for the full activation of the IGF-IR, which would explain the EPO-independent IGF-I hypersensitivity of PV.

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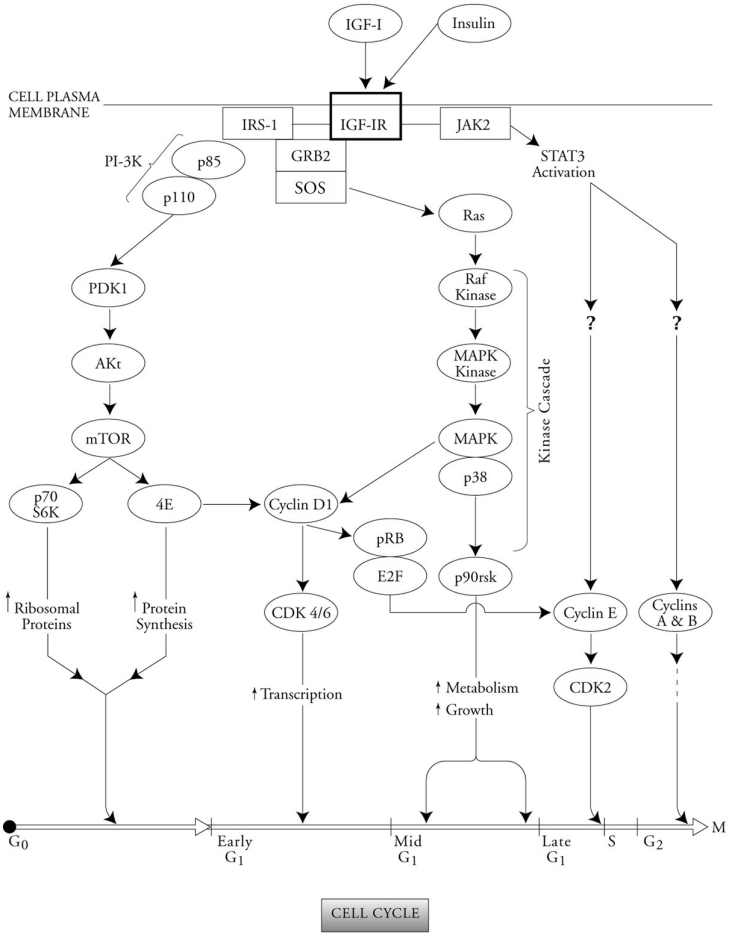

Fig. 22 - Insulin-like Growth Factor I receptor (IGF-IR) signaling and control of cell cycle, including the JAK2 interaction with *STAT3*. For the modulation of the IGF-I signal by *SOCS* genes see **Table 10**.

4. A unitarian, growth factor hypersensitivity model of the CMPDs

4.1. The CMPDs

In 1971, Ward and Block separated, on the basis of their clinical studies, the acute myeloid leukemias (AML, AEL) from the myeloproliferative syndrome (MPS, then the name of the chronic myeloproliferative disorders, CMPDs), which was composed by PV, ET and idiopathic myelofibrosis (IMF, now MMM, myelofibrosis with myeloid metaplasia, or MDS) [114]. Then, in 1983, Axelrad's group showed that the CMPDs were proliferative disorders of more restricted progenitor cells than those responsible for the acute leukemic blastoses, and that the differences between CMPDs resulted from the lineage-specificities of these restricted progenitors [115].

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4.2. Altered JAK/STAT signaling and cytokine hypersensitivity in the CMPDs

JAK, the gene for Janus kinase, belongs to a family of tyrosine kinases that work intra-cytoplasmically to bind and phosphorylate the cytoplasmic portion of various cell surface receptors, so as to permit them to trigger and regulate the signal transduction cascade that controls the metabolic and transcriptional response of cells to growth and differentiation factors. In embryonic erythropoiesis, JAK2 deficiency (*JAK2*-/-) abrogates expression of BFU-E and CFU-E, and leads to anemia and death, but without altering the $CD34^{low}$ CD117+ compartment [116].

Constitutional JAK-STAT signaling due to fusion of the transcriptional factor gene *TEL* to *JAK2* was first reported in B cell precursor ALL and in CMML [117-118]. STATs ("signal transducers and activators of transcription") are a group of cytoplasmic latent transcription factors (proteins) that serve as substrates for JAK and, once phosphorylated, move to the nucleus where they mediate growth factor-directed transcription. Normally, activation of the STAT protein requires activation of JAK, but in CML, the *bcr-abl* gene fusion product (see below) directly phosphorylates the STAT5 protein independently of JAK [119]. This results in the translocation of STAT5 to the nucleus where it activates a variety of functions that essentially block apoptosis and result in growth factor independence associated with neoplasia. In transformed cell lines, STAT3 is commonly activated by the IGF-IR [120]. Activated STAT3 is sufficient to induce cellular transformation [121], and it has been shown that STAT3 is constitutively activated in granulocytes of some PV patients [122], suggesting it may be involved in the promotion of granulocytosis in these patients.

Several groups of investigators reported that hematopoietic cells of CMPD (chronic myeloproliferative disorder) patients possess a single gene mutation in common [123-126]. This is the clonal, recurrent, activating somatic mutation in the SH2 pseudokinase auto-inhibitory domain of the *JAK2* gene found in PV patients. It results in a guanine to thymine substitution in *JAK2* exon 14, that leads to a valine to phenylalanine substitution at amino acid position 617 and to constitutive activation of the JAK tyrosine kinase. The mutation was detected in granulocytes, but also in erythroblasts differentiated from CD34+ cells (likely CFU-GEMM), and in platelets but not in T cells. It was found in a large majority of patients with PV, and in a significant number of patients with ET and MMM/MDS. Constitutive activation of STAT proteins is frequently found in tumor cells or derived cell-lines, and abnormalities of the JAK/STAT pathway are associated with other types of human cancer (eg breast carcinoma) [127-129]. In nude mice, the mutation has been shown to be tumorigenic [130].

Remarkably, inhibition of JAK/STAT signaling results in suppression of cancer cell growth, and induces apoptosis in various cancer cell types [129]. Zong et al have reported that STAT3 is activated in response to IGF-I/IGF-IR stimulation *in vivo* and *in vitro*, and that STAT3 activation can be inhibited by SOCS proteins [120]. This suggests that aberrant JAK/STAT signaling may play an

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important role in malignant progression by regulating cell growth and survival.

The foregoing presents the JAK/STAT signaling circuit as a preferred target for leukemias (myeloid and lymphoid), somatic cancers and the CMPDs. Moreover, the fact that all three CMPDs share a single JAK2 mutation provides the molecular link that unifies them. However, it does not account for the differences between their phenotypes.

We investigated the growth-factor responses of the CMPDs with the carefully controlled, practically defined, serum-free culture method that we developed (the Correa-Axelrad "SeroZero" medium [62, 66, 131-132]). PV erythroid progenitors (BFU-E) were shown to be exquisitely sensitive to IGF-I [88] but not to EPO [88], thrombopoietin (TPO) [133], Megakaryocyte Growth and Development Factor (MGDF) [133], GM-CSF, IL-3 [134], nor to SCF (ligand of CD117, the c-*kit* gene product) [98].

Conversely, the megakaryocytic progenitor cells of ET patients were found to be hypersensitive to MGDF and TPO, but not to IGF-I, Epo, IL-3, GMCSF and SCF [133]. Finally, megakaryocytic progenitor cells in myelofibrosis with myeloid metaplasia (MMM/MDS) were hypersensitive to SCF, but not to IGF-I, Epo, IL-3, GM-CSF, FGFb and PDGF [92, 134].

The following **Table 9** summarizes the genomic JAK/STAT alterations in CMPDs and leukemias:

Table 9

CMDPs:	
•PV	*STAT 3*: Constitutive activation in granulocytes;
	JAK2 mutation: *JAKV617F* in:
	erythroblasts, megakaryoblasts & granulocytes
•ET	*JAK2V617F* mutation
•MMM (IMF)/MDS	*JAK2V617F* mutation
Leukemias:	
•CMML	*TEL*/*JAK2* fusion: constitutive JAK/STAT signaling ;
	bcr-abl encoded P210 kDa binds to STAT5
•CML	*TEL*/*JAK2* fusion
•preB-ALL	*TEL*/*JAK2* fusion

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4.3. IGF-I hypersensitivity, the JAK/STAT pathways and the SOCS genes

Another factor, besides the JAK2V617F mutation, contributing to the IGF-I hypersensitivity of PV circulating progenitors could be a defect in negative regulation of cytokine activity. The activities of cytokines are influenced by a variety of negative regulators. Important among these are suppressors of cytokine signaling (SOCS) [136]. SOCS have been shown to be negative regulators of cell population size by inhibiting cytokine receptor signaling via JAK/STAT or other pathways [111, 137]. A partial list of the signaling proteins with which they associate and the cytokines whose signaling they inhibit is shown in **Table 10**.

The SOCS family consists of eight proteins (SOCS 1 to 7 and CIS) containing a central src-homology (SH2) domain which functions in gene regulation by recognizing different targets in the cytokine receptors and/or their kinases, and a specific 40 amino acid protein motif at the C-terminal end (SOCS box) [138-139]. In some cases, this motif is involved in gene regulation via the ubiquitin mechanism of protein degradation [140-141]. Transcripts encoding SOCS-1, SOCS-2, SOCS-3, and CIS-1 are often present in normal cells at low levels, but they can be rapidly increased in response to a wide variety of cytokines, growth factors, inhibitory proteins, or hormones [139, 142]. Overexpression of any of the SOCS proteins in a physiological setting results in inhibition of cytokine signaling [139].

SOCS-1 is critical in the negative regulation of IFNg signaling, mediates IFNa repression of megakaryopoiesis [143] and in the differentiation of T cells. SOCS-1 also associates with JAKs to inhibit their catalytic activity

SOCS-2 is believed to mediate a normal function of negatively regulating IGF-IR signaling. SOCS-2 protein interacts strongly, both in vitro and in vivo, with the IGF-IR when it is activated

Table 10

SOCS family members associate with a variety of signaling proteins and inhibit signaling by cytokines

Name	Inhibits signalling by	Associates with receptors and proteins
CIS	IL-2, IL-3, prolactin, Epo, IGF-1	Epo, IL-3. GH, IL-2
SOCS1	IL-2, IL-3, IL-4, IL-6, GH, prolactin, Epo, IFN, Tpo, IGF-1	JAK1, JAK2, JAK3, Vav, FGF, GH, IGF-1, PYK2
SOCS2	GH, IL-6, LIF, IGF-1, prolactin	IGF-1, prolactin, GH
SOCS3	IL-2, IL-3, IL-4, IL-6, GH, Epo prolactin, LIF, IFN, IGF-1, insulin	FGF, PYK2, GH, Epo, leptin, IGF-1

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(tyrosine phosphorylated) to inhibit IGF-I signaling, but not with a kinase-negative mutant of this receptor [144]. SOCS-2 is also known to inhibit signaling by GH [145]. A defect in *SOCS-2* gene expression might be expected to promote IGF-IR signaling; it might also promote the anti-apoptotic (accumulative) function of IGF-I, both possibilities resulting in an increase of erythroid cell number.

At a specific stage in fetal hematopoiesis, *SOCS-3* becomes highly expressed, apparently to negatively regulate the size of the erythroid compartment [146]. Transgenic mouse embryos that constitutively overexpress *SOCS-3* lack fetal liver erythropoiesis [146]. Evidently the suppression of cytokine signal transmission overrides cytokine stimulation at a critical moment for the expansion of the erythroid compartment. Conversely, embryos that are *SOCS3-/-* die from what appears to be a massive erythrocytosis occurring throughout the body. However, this was not confirmed by a subsequent study, which claimed SOCS-3 to be dispensable for normal hemopoiesis in the mouse embryo [147].

The emerging model for the regulation of signaling at the level of a cytokine receptor (see **Fig. 23**) is that JAK intracytoplasmically associates with a variety of these receptors, in particular with the IGF-IR, permitting activation of signaling when JAK phosphorylates STAT and releases it from the receptor. Dimers of STAT enter the nucleus and activate transcription of the *SOCS* and *CIS* genes. In turn, the SOCS and CIS proteins engage in the negative regulation of signaling. SOCS-3

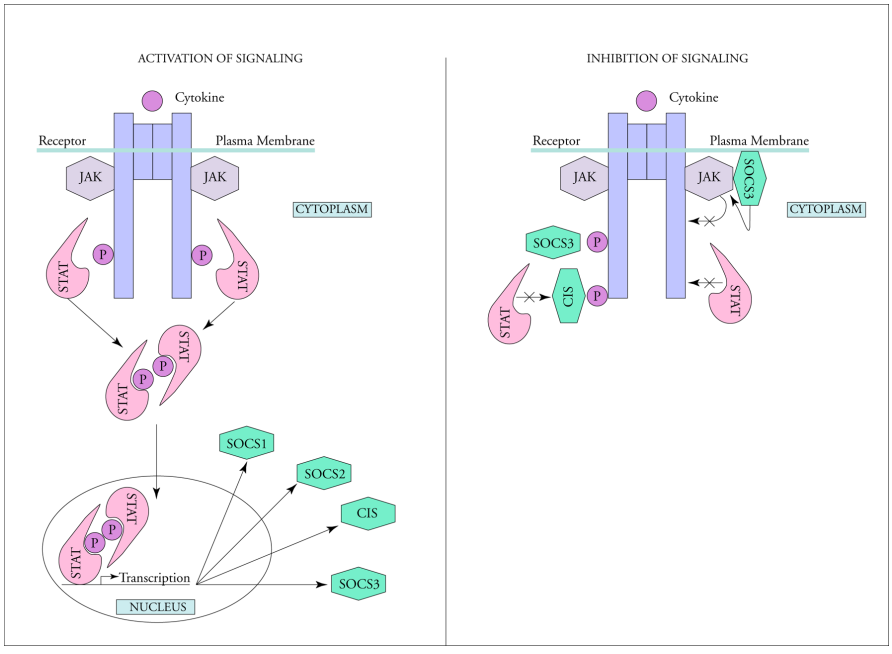

Fig. 23 - Model of STAT- and SOCS-mediated positive and negative regulation of cytokine-receptor signaling by Janus kinases (JAKS).

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and CIS proteins in the cytoplasm now bind to the cytokine receptor, block STAT from binding to it, with SOCS-3 also binding to JAKs and inhibiting their kinase activity.

The implications of the above for the understanding of PV - and neoplasia - are profound. Hematopoiesis is a delicately balanced process regulated *positively* by growth and differentiation cytokines, and *negatively* by suppressor genes and their protein products, at all stages of blood cell development. Any "autonomization" of either control arm - such as independence from a growth factor, or a constitutive expression of a suppressor gene (such as *STAT3* or *SOCS-3*), whether mutated or not - can derail hematopoiesis and give rise to an oncogenic or pro-oncogenic disorder. Thus, together or separately, defects in *SOCS-2* and *SOCS-3* gene expression could lead to an increase in erythroid cell population size in PV.

Theoretically, the reduced expression of SOCS-2 and SOCS-3 in PV alone could be the cause of this neoplasm. But in vitro, with the Correa & Axelrad serum-free medium, we showed that the reduced expression of these genes in PV cells occurred only in the presence of IGF-I [113]. In other words, the reduced expression of SOCS-2 and SOCS-3 was part of the response to IGF-I. In contrast to PV patients, in erythroid colonies derived from healthy donors, IGF-I promoted an increase in the expression of SOCS-2 and SOCS-3 genes [113].

However, there seemed to be a limit to the suppression by SOCS-2 and SOCS-3 of IGF-I activity in stimulating erythroid colony formation. At some level of SOCS suppression of IGF-I activity, erythroid colony formation, though reduced, could still go on independently of IGF-I [113]. Previously we had observed that most patients did not produce EECs and EEBs in the absence of EPO and IGF-I, when only hemin and IL-3 were added (see, for example, figure 1 of [88]), even if retinyl acetate (RA) or all-*trans*-retinoic acid (ATRA) was introduced (at 30nM final concentrations). But we had also noticed that some PV patients still presented substantial EEB formation under the same conditions. In fact, an example of this can be found in **Fig. 12A** (columns 1 and 2), and there - in the presence of RA - it is apparent that an EPO-independent *and* IGF-I-independent erythroid burst formation is still ongoing.

Moreover, in the absence of IGF-I, overexpression of the *SOCS2* and *SOCS3* genes in transfected PV mononuclear cells was still able to reduce the number of erythroid burst-component colonies [113]. Because it occurred in the absence of IGF-I, we called this an IGF-I-independent ('non-specific') reduction in erythroid colony numbers. Most likely SOCS2 and SOCS3 were able to suppress the action of another cytokine that could promote erythroid colony formation. IL-3 would be a possible candidate, even though in serum-free culture PV cells are not hypersensitive to IL-3 (see **Fig. 14**) [134]. It is known that the activity of IL-3 (like IGF-I) is regulated by SOCS family proteins [138].

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Another candidate is IL-11, which is apparently higher in serum and bone marrow plasma of PV patients than normals [148]. Though unclear whether IL-11 is produced by either stromal cells [148] or CD3+ T cells [149] in PV, IL-11 may play a role in erythropoiesis, since anti-IL-11 antibodies could decrease some burst formation [149]. Burst formation by CD34+ cells of PV peripheral blood could be stimulated by conditioned medium from PV CD3+ T cells, and the responders were positive for the JAK-2 mutation [149]. Yet, IL-11 is normally not produced by CD3+ T cells, nor has its effect been tested in serum-free culture. Furthermore, the presence of other cytokines could not be ruled out in the conditioned medium preparation.

A more likely candidate, in our view, is the retinoid (vitamin A) axis that has been implicated in normal myeloid and erythroid differentiation [63, 150]. However, it has been reported that high concentrations of ATRA (10 µM, equivalent to suprapharmacological concentration) inhibited erythroid colony formation by PBMNC from seven PV patients [151]. But this finding suffered from two basic insufficiencies. First, the authors employed a commercial, so-called "serum-free" medium (MethoCult SF H4436; Stem Cell technology, Vancouver, BC, Canada) that is *not* truly serum-free [152]. This will skew the lower part of any ATRA titration curve. Secondly, and far more importantly in terms of the hurried therapeutic recommendations made by Steidl et al [151], Correa and Axelrad showed that in a true serum-free medium, ATRA concentrations as low as 0.3µM significantly decrease normal erythroid colony and burst formation by PBMNC from *normal* donors [63]. At 30µM ATRA, we found virtually no erythroid colony formation [63] (see **Fig. 24**). Thus, the *same* inhibitory effect of high ATRA concentrations *is observed in normals as in PV*, and it must be concluded that it is *merely toxic*. Steidl et al seem to have been oblivious to these findings made some 13 years earlier. Nor did they carry out controls with normal mononuclear cells that would have shown the same toxic inhibitory response [151].

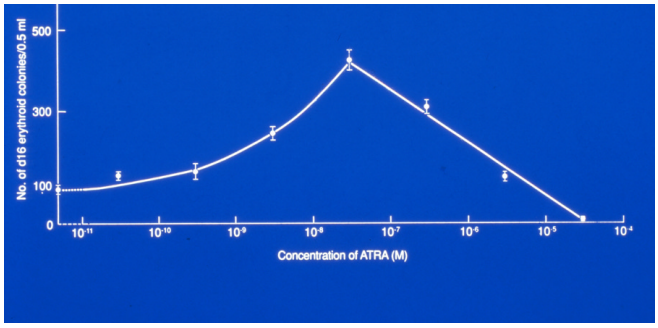

Fig. 24 - Erythroid burst-component colony (BCC) formation by normal circulating erythroid progenitors in a true serum-free medium, as a function of ATRA concentration, and in the presence of rHu EPO, rHu IGF-I, hemin and IL-3. Above 0.03µM ATRA, the tested concentrations of the retinoic acid are toxic and thus inhibit erythroid colony formation. At 30µm ATRA virtually no colonies are observed.After Correa & Axelrad [63].

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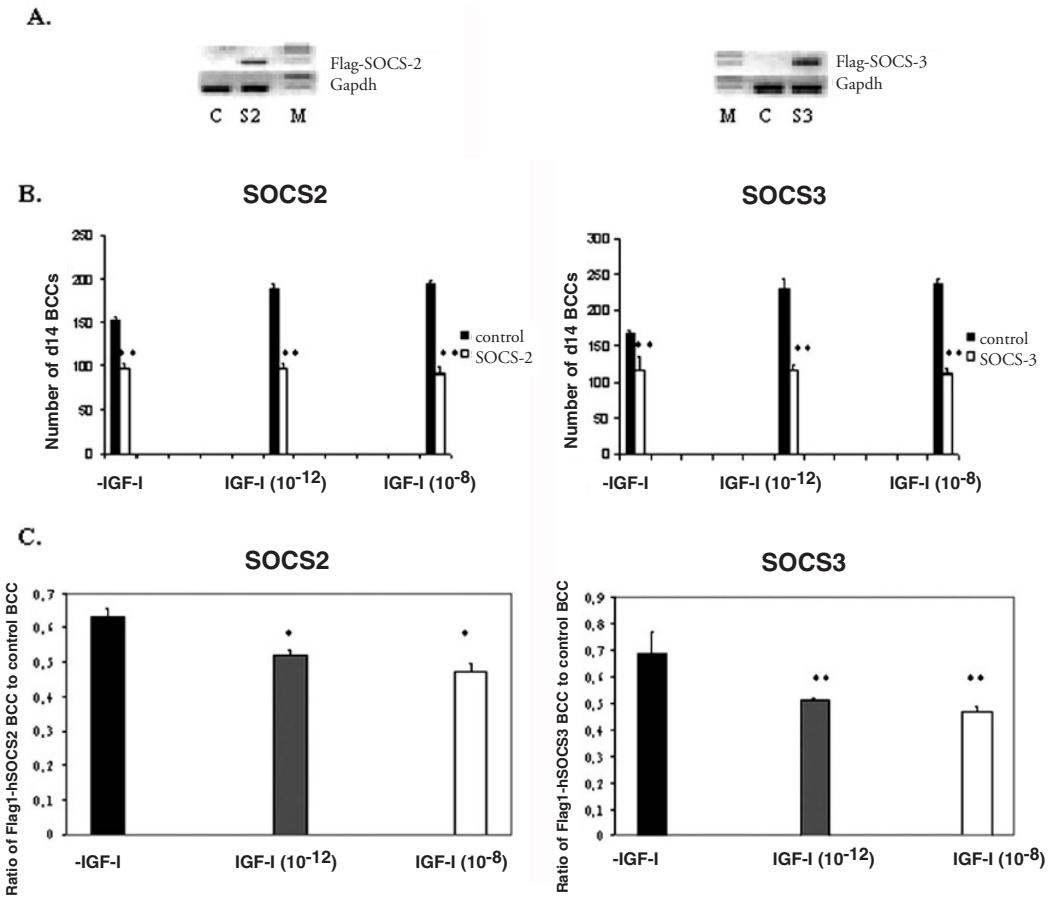

Fig. 25 - Effect of overexpression of *SOC2-2* and *SOCS-3* genes by transfected PV mononuclear cells upon PV erythroid colony (d14 BCC) growth, in the presence and absence of IGF-I. (**A**) Expression of Flag-*SOCS-2* and Flag-*SOCS-3* in transfected PV eryhroid cells (GAPDH was employed to prove that transfection was successful). (**B**) Observed reduction in erythroid colony (d14BCC) formation by transfected PV mononuclear cells overexpressing *SOCS-2* or *SOCS-3*, in the presence and absence of IFG-I. (**C**) IGF-I-specific reduction of d14 BCC produced by *SOCS-2*- or *SOCS-3*-overexpressing cells. Results are expressed as ratio of the number of BCCs following transfection of the *SOCS-2* or *SOCS-3* vectors, to the number of BCCs following transfection with empty (control) vectors, and reported as the mean ±SEM of triplicate wells of the same experiment (*P<0.02, and **P<0.05). After Usenko et al 2007[113].

Accordingly, of greater interest, is the unresponsiveness of PV mononuclear cells to low, *non-toxic* concentrations of ATRA, and the possible relation this may have with the expression of EPO-independent *and* IGF-I-independent erythroid colony formation in *true* serum-free media. In this sense, it is curious that the same German study also found that CD34+ cells from PV patients

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overexpressed all three retinoid receptors [153] - RAR-beta (retinoic acid receptor beta), RXR-beta (retinoid X receptor beta) and CRABP2 (cellular retinoic acid binding protein 2) - even though it did not suggest any molecular process that might explain the finding. We should note that addition of a RAR antagonist significantly decreases *SOCS3* mRNA expression, and addition of retinoic acid induces *SOCS3* expression - and that these processes are implicated in the regulation of auto-immunity [154].

It is possible, therefore, that PV presents different degrees of refractoriness in the response of its mononuclear cells to retinoids. At physiological retinoid concentrations, retinoid-responsive PV PBMNC would be able to generate a modicum of EPO-independent *and* IGF-I-independent erythroid colonies in true serum-free media. Retinoid-nonresponsive PV PBMNC would not be able to do so. Only the retinoid-responsive PV PBMNC could be further suppressed by SOCS 2 and SOCS3 in the absence of IGF-I. That would suggest that a progressive internal blockage of the retinoid response could be in play. Since PV apparently presents no cytogenetic abnormalities on chromosomes 3p24 (*RAR-beta* gene), 6p (*RXR-beta*) and 17 (*RAR-alpha*), it seems that the anomalous retinoid response (the hypothetical vector that progresses from retinoid responsiveness to unresponsiveness) will likely reflect a transcriptional upregulation of the retinoid receptor genes. This could be an epigenetic response to a RAR/RXR antagonist capable of capping the retinoid receptors and consequently decreasing *SOCS3* mRNA expression. Such an antagonist would probably stabilize the co-repression complex of the RAR-alpha/RXR- heterodimer. Increased presence of such an antagonist would make the PV mononuclear cells progressively more unresponsive to retinoids. In turn, this incremental unresponsiveness could further accentuate the acquired IGF-I hypersensitivity (which we now know can reach several orders of magnitude greater than normal sensitivity).

Lastly, overexpression of SOCS-2 and SOCS-3 in transfected cells from PV patients reduced the increased colony number brought about by IGF-I (thus counteracting the ability of IGF-I to stimulate erythroid colony production), and decreased the sensitivity of the cells to IGF-I [113] (see **Fig. 25**). Thus, overexpression of SOCS-2 and SOCS-3 genes reversed the IGF-I hypersensitivity and erythroid overgrowth, both characteristics of the PV phenotype in cell culture.

5. Myelodysplastic syndromes (MDS) and leukemia

5.1. Is MDS an hyperplastic "preleukemic" syndrome or a leukemia?

MDS is, much like AIDS, an "impure" syndrome. One may argue that the syndrome is wider in scope than either IMF or MMM, but more and more it seems that these are just different names for the same general process of fibrotic and dysplastic disturbance of marrow, typically accompanied by extramedullary hematopoiesis. As discussed above in the anemia section with respect to refractory

 ISSN 1920-3799

manifestations, IMF/MMM/MDS implicates a myeloid stem cell, as well as a fibrocytic response to what is the breakdown of the marrow hematon structure. MDS-type responses are encountered in exposure to benzene or ionizing radiation, and the current medical view is that its incidence, though admittedly unknown, is now more frequent than in the past, partly due to an increase in treatment-induced (iatrogenic) leukemias. As discussed already, in parallel with myelophtisic anemia, extramedullary hemopoiesis causing splenomegaly and hepatomegaly is observed in MDS. Ineffective hemopoiesis produces a gamut of cytopenias. These typically affect erythropoiesis first, so that the early MDS stages present anemia (with or without sideroblasts, see **Table 3**), despite either normal marrow cellularity or hypercellularity associated with erythroid hyperplasia. The blast percentage of MNC in marrow is low (<5%), but if sideroblastosis is present, it is typically greater (<15%). As MDS progresses, the percentage of marrow blasts increases (up to 20%) - often showing up in peripheral blood - and two or more cell lineages are affected with cytopenia. Some degree of thrombocytopenia is usually observed, and the granularity of neutrophil cytoplasm is typically abnormal.

As discussed above, we suggest that MDS has early and late phases, and the fact that PV often evolves into forms of myelofibrosis easily leads one to wonder just what is the difference between the marrow erythrocytosis characteristic of MDS early-stage hypercellularity and the IGF-I hypersensitive erythroid hyperplasia characteristic of PV. Progression of MDS towards CML, acute myelogenous leukemia (AML), and frequently towards AEL (AML/M6), occurs in nearly half the cases, and variants such as chronic myelomonocytic leukemia (CMML) are observed where the percentage of blasts is excessive and blood monocytosis is present. The realization of these various facts led understanding of myelofibrosis prior to the 1980's to regard it sometimes as an erythroid leukemia, one that spanned from chronic erythrocytosis and myelocytosis to acute erythroblastosis and myeloblastosis. Gunz pointed out that "chronic forms of erythroleukemia" were virtually identical with the refractory anemias, including RARS [155], "the distinction [from the latter] being evidently a fine one" [156]. He also pointed out that, while the erythroblastosis characteristic of myelofibrosis tended to evolve towards a nonerythroid form of AML (back then called acute granulocytic leukemia), and acute myelofibrosis was "analogous to acute leukemia", it was unclear whether "this is a 'natural' sequel or possibly a complication of therapy" [157].

Cytogenic abnormalities involving chromosomes 5 and 7 are frequently reported in MDS. There is no established treatment for MDS. In ca. 25% of the cases, treatment with growth factors (EPO, G-CSF, GM-CSF and TPO) increase production of RBCs, granulocytes, monocytes and platelets, but "survival advantage has not been shown" [158]. Moreover, given that the blast percentage of MNC increases with the aggravation of the disorder, treatment with growth factors, even differentiation-promoting ones (like EPO), is likely to enhance, rather than correct, myeloid proliferation,

 ISSN 1920-3799

and thus hyperplasia. Conversely, in late MDS stages, the growth factor treatment is ineffective due to the pervasive cytopenias (no more hematopoietic progenitors to recruit that may commit to differentiate). The cytopenias are aggravated by marrow fibrosis in hypocellular stages typically associated with the onset of AML [159].

5.2. MDS evolution into acute leukemia

Sarkodee-Adoo et al [160] list the common limitations of purely morphologic systems of classification of leukemias and hematopoietic disorders. They itemize a series of negative traits that can easily condemn a diagnostic as arbitrary and nonscientific. In our view, the gravest are (1) dependence on observer (physician, researcher) judgement - which Sarkodee-Adoo et al identify with lack of reproducibility - and (2) "frequent dependence on arbitrarily established categories", in turn founded on purely morphological and/or staging criteria (upon which decisions made by technobureaucrats, viz WHO, have a considerable impact). The MDS classification and a better understanding of the disease have suffered from both these limitations. Moreover, it seems that the "syndromes" have not been adequately grasped as being *both neoplastic and "preleukemic"* (pre-AML or pre-CML) - and thus are not regarded as stages of an auto-oncogenic vector ultimately leading from a chronic neoplasm to acute leukemia. Gunz underlined the possibility of such a vector or sequel, when he commented that, in this respect, the difficulty facing understanding of refractory anemias and RARS was "that some of them might eventually have developed frank erythroleukemia if their course had been long enough" [156]. The relative rarity of the score of erythroleukemia is likely also due in part to the difficulty in staining and recognizing neoplastic erythroblasts, in particular in distinguishing them from lymphoblasts on the basis of morphological criteria alone.

"The hallmark of MDS" - write Sarkodee-Adoo et al - "is the presence of peripheral blood cytopenias in the setting of bone marrow hypercellularity and dysplasia" [160]. The intensity of the dysplasia renders the generated erythroid cells ineffective, resulting in "universal anemia". The erythrocytes are initially macrocytic but once the ringed sideroblast stage is reached, anisocytosis becomes pronounced, with erythrocytes that are increasingly microcytic.

Yet, the hallmark of MDS also appears to be that it constitutes a stage in a leukemic/oncogenic vector. Here, too, the "reliance with a blindfold" (to use the expression of Sarkodee-Adoo et al) on 'statistics-rendered-meaningless' has blocked understanding of the continuously-evolving connection with a neoplastic disease vector. The fact that only half (again, 40 to 60%) of the MDS cases evolve into leukemia obscures the fact that that most who are diagnosed with MDS do not have a chance to live long enough (due to marrow fibrosis and sclerosis) to endure its conversion into myeloid leukemia. Moreover, the inclusion of CMML inside the MDS group of syndromes (a move which the 2008 WHO reclassification of blood neoplasms abandoned) forces us to conclude that, at

 ISSN 1920-3799

least, 55 to 75% of all MDS cases include either chronic leukemia or evolve into acute leukemia. MDS appears to be an already malignant disorder, at least parallel in staging to the chronic leukemias and the CMPDs [161]. This contention is emphasized by the finding that 50% of the patients affected by refractory anemia with ring sideroblasts (RARS) in marrow present ET features, with 31% carrying the JAK2 *V617F* mutation [162].

Harping further at inconsistencies in disease diagnostic criteria, Sarkodee-Adoo et al underlined how ad hoc (their very words are "arbitrarily selected") are the bone marrow MNC percentages of blast cells allocated and employed to separate MDS from AML [163]. If a blast percentage on the order of 20% were used as criterion to distinguish MDS from AML, ca 35% of MDS would fall under the AML rubric. Refractory anemia, with or without sideroblastosis (see **Table 3**) would then be regarded as the essence of the (singular) myelodysplastic syndrome since the 15% belonging to CMML would be equally allocated to another leukemia (and, at once, to one that is difficult to differentiate from AML). Remarkably, 93% of patients with RAEB-t (refractory anemia with excess of blasts in transformation, see **Table 3**) express CD117 [164], the SCF receptor, indicating that very primitive myeloblasts are involved, and once again suggesting that the disease could be regarded as being already leukemic or fully neoplastic.

Sarkodee-Adoo et al inclined to a continuum distribution of blast counts linking different "clinical outcomes" (we would have preferred to say - different "clinical phases" of the neoplastic disease vector). We also do not believe that the onset of leukemia can be determined by a fixed PMC-blast % threshold, all the more so as the increase in blast number will form a continuum. Further, it is apparent that some myeloid stem cell, with some degree of hemopoietic pluripotentiality, has been recruited to respond to what is a persistent stress condition, at some critical juncture of stress intensification. Likely, the leukemic transformation will affect a stem cell in the original hyperplastic blast clone. It is in this sense that Kathryn Foucar writes: "High-grade myelodysplastic disorders and AML may represent a biological continuum that likely results from a stepwise accumulation of genetic lesions following the initial development of clonal [dysplastic] hematopoiesis" [165].

If we vectorialize the connection between MDS, refractory anemia and acute myeloid leukemia, we arrive at a process that begins with a complex of factors affecting erythropoiesis (the body's energy system) and resulting in anemia, and which evolves - through dysplastic and metaplastic stages - towards acute erythroleukemia associated or not with other forms of AML. A recent study suggested that MDS-associated erythroleukemia (AML-M6a) should be differentiated from a "true erythroleukemia" (AML-M6b) because of its multilineage involvement [166]. The same study found that half of the erythroleukemic patients had a history of MDS.

From the above, we can provisionally conclude to an auto-oncogenic vector for the myelodys-

 ISSN 1920-3799

plastic syndromes initiated by multilineal hyperplasia and dysplasia with an erythroid emphasis:

MDS —> Macrocytic Anemia —> Sideroblastosis —> Microcytic Anemia —>AML, AML-M6a
[<————————————REFRACTORY ANEMIA————————————>]

We note that the conversion of the vector into forms of AML may cross another intermediary stage that is essentially linked to the expression of Ph+ CML [167]. However, just as we have suggested and discussed above regarding the disturbed immunological relationship between CLL and AIHA, it is likely that the etiology of MDS is auto-immune, and thus that the oncogenic vector involves an initiation process parallel to that which we showed above for AIHA. Auto-immune hemolytic responses have been found associated with MDS [168-169], suggesting that, once again, in MDS presenting refractory anemia, the initiation vector may be as for AIHA above:

increased RBC lability —> T cell dysregulation —> anti-RBC autoreactive B cell clone —> MDS

Of course, from a mechanistic perspective, one could begin from the T cell dysfunction without inquiring about what it responded to. From a functional perspective, however, this begs the questions of the adaptive response of regulatory T cells, and of what are the dominant factors affecting RBC lability that make its differentiation dysplastic to begin with. It is equally possible that in MDS presenting refractory cytopenias or pan-cytopenia, the auto-immune mechanism may be further deregulated than simply aiming at the erythroid compartment, even if its initial target is erythroid self-tolerance.

6. PV and the CMPDs as hematologic neoplasms and their relation to leukemia

6.1. Polycythemia *vera* as a hematologic neoplasm

In light of the preceding sections, several criticisms can now be addressed to the improved staging model of PV (**Table 8**). These criticisms, however, are also subject to counter-arguments - which we will equally present below. Jerry Spivak introduced these criticisms to the revised natural history of PV by making a sharp observation that is long-overdue:

"Attempts to define the natural history of polycythemia vera then as now have been frustrated not only by the low incidence of the disorder but also its chronic nature which precludes most physicians from seeing more than a few of these patients or even following them for a sufficient duration to encounter the full scope of the disease. These factors coupled with an initial lack of appreciation of the effect of radiation or chemother-

 ISSN 1920-3799

apy on bone marrow function led to the acceptance of anecdotal case studies as representative of the natural history of polycythemia vera." [170]

Spivak raises here two distinct difficulties that led to the creation of a mythical natural history for PV - one is a diagnostic difficulty inherent to the slow progress of PV, and the other a clinical difficulty posed by iatrogenic artifacts in the uncritical treatment of PV. We should note that Spivak's approach treats PV as a malignancy or neoplasm in its own right, just not one that is leukemic or necessarily evolves into a leukemia. It is important to retain this, because what follows fundamentally agrees with Spivak that PV is a neoplasm - while disagreeing with Spivak, in that we will contend that PV is a myeloproliferative (and not myeloaccumulative) disorder equivalent to a chronic leukemia, and that it belongs to an auto-oncogenic vector that tends towards acute leukemia, irrespective of iatrogenic distortions of this tendency.

But let us examine in some detail the fundamental criticisms of the paradigm of the natural history of PV, together with their counter-arguments or qualified objections:

1) The marrow hyperplasia in PV is truly trilineal, or myeloid, and not just erythroid. The disease is clonal, and the marrow heterogenous at first.

From a neo-lamarckian perspective [1], the abnormal clone that is hyperplastic is elicited to respond to the selecting auto-oncogenic pressure - a cyanosis with depressed EPO concentration that affects the erythroid lineage above all. It is this acquired emphasis that drives the myeloid hyperplasia towards EPO-independent erythropoiesis, and thus runs the risk of depleting the iron stores and causing what is often the first overt symptom of PV - a iron-depletion microcytic anemia. Thus, the fact that the hyperplasia is pan-myeloid does not obfuscate its erythroid emphasis, and it is this emphasis alone that defines the PV-specific phenotype.

2) There is no evidence that the RBCs differentiated from the hyperplastic clone are in fact normal at any time, as the natural history has assumed. Progression of the disease is frequently associated with increased Hb F expression by the cells of the hyperplastic clone [171]. The splenomegaly attendant to PV clearly indicates that some degree of erythroid dysplasia or dyserythropoiesis must be at work. Progressive shortening of RBC life-span is observed, but is thought to be secondary to increasing splenic sequestration of deficient RBC's with abnormal morphology [172-174]. Irrespective of order of causation or dominance (whether it is the erythrodysplasia that leads to the splenomegaly, or the splenomegaly that leads to the erythrodysplasia), when the disease progresses to the so-called "spent phase", poikilocytosis in marrow and peripheral blood becomes a marked trait, among a

 ISSN 1920-3799

profusion of ovalocytes.

Of course, the question of whether erythroid differentiation is or not normal in the early stages of PV begs the question of whether a covert phenotype like that of IGF-I-hypersensitive erythroid progenitors in PV is a marker of an erythrodysplastic, but non-morphologically detectable, differentiation. This question is linked to the still commonly held prejudice that the EPO response in PV erythroid progenitors is not normal. The hyperplastic clone is effectively independent of EPO because it is hypersensitive to IGF-I, but this does not mean that its response to EPO is not normal, which it is (just as we have shown above at length is the case). Thus the RBC differentiation is entirely carried over by the hypersensitive response to IGF-I, and it is this that is likely linked to some form of anaplasia (since IFG-I hypersensitivity constitutes some form of a return to an embryonic-type of erythroid response, if not a 'biomnemic' recurrence of the embryonic response) and the adaptive reversion to Hb F production.

3) If sufficient iron is given to PV patients, microcytic anemia as a result of PV is uncommon, as Spivak has underlined. Spivak suggests other anemias have different causes, including iatrogenic ones [170]. In fact, phlebotomy is one of the main factors aggravating the iron loss.

Yet, some PV-associated anemias cannot be corrected with iron supplements, because they are of the pernicious type caused by vitamin B_{12} or folate deficiency (and likely entailing a vitamin C deficiency as well), or of the refractory type associated with the progression of PV. The pernicious forms are likely connected to increased B_{12} binding affinity of PV serum, and appear to be intrinsic to the disease phenotype. This also raises the question of possibly disturbed self-tolerance.

4) Spivak also reminds us that the evolution of the PV condition towards MDS-like symptoms may equally be iatrogenic. Spontaneous regression of myelofibrosis has been observed [175-179], and the incidence of myelofibrosis is double in PV patients that have been exposed to ionizing radiotherapy or chemotherapy, when compared to those treated with phlebotomy.

However, in this respect, Spivak's approach fails to address what appears to be the core of the relation between PV and myelofibrosis, specifically qua myelodysplasia (MDS). The *JAK2* mutation common to all CMPDs appears to be sufficient to induce myelofibrosis (see above), and is implicated in myeloid hyperproliferation. Moreover, PV frequently exhibits increased marrow reticulin and other symptoms of MDS at presentation. Disturbance of the hematon structure and function involves a myeloid stem cell that is likely the same in PV and MDS, and IGF-I-activated fibroblasts are probably involved in the fibrocytosis associated with MDS. Spivak accepts that increased marrow reticulin may be an integral part of the natural history of PV, but he doubts whether myeloid metaplasia or

 ISSN 1920-3799

other features of MDS legitimately constitute a late feature of PV.

5) In the absence of radiation or chemotherapy, splenomegaly does not necessarily correlate with extramedullary hemopoiesis. Spivak is entirely correct in this criticism.

In accordance with our views presented in the preceding and following communications, it is likely that initial splenomegaly actual reflects an hemolytic process caused by the increased poikilocytosis of circulating RBCs, and possibly by an heretofore unstudied and unremarked echinocytosis (see following communication) or eryptotic tendency of the RBCs towards fragmentation.

However, this, of course, does not mean that extramedullary hemopoiesis (myeloid metaplasia) may not be an outcome of the PV disease vector, nor that it will not later contribute to splenomegaly.

6) There is no observed correlation between marrow failure (whose real frequency in PV is still unknown) and the occurrence of extramedullary hemopoiesis (essentially splenic myeloid metaplasia) [177]. It is possible and likely that the PV cases that develop myeloid metaplasia are iatrogenic.

We think that this criticism of the natural history of PV may turn out to be incorrect. There may well be no known correlation between marrow failure and extramedullary hemopoiesis for PV patients that have *not* undergone ionizing radiation or chemotherapy. Yet extramedullary hemopoiesis, whether iatrogenic or "naturally occurring", typically accompanies or follows marrow failure. Myeloid metaplasia may be a basic feature of MDS, yet, in our view, this does not rule out the likelihood that this feature is mobilized to compensate for marrow failure in MDS or in other conditions that destroy the marrow.

7) Cytotoxic agents, such as those used in chemotherapy, as well as ionizing radiation, promote myeloid metaplasia, and correlation of extramedullary hemopoiesis with patients exposed to either "treatment", if it were ever made, may also point to an iatrogenic effect.

Again, the criticism has a backbone of validity, but, as with the previous entry point, we should note that ionizing radiation or chemotherapy may well induce, as they do, myeloid metaplasia, marrow fibrosis and anemia; yet, that does not mean that in their absence - and undoubtedly in a different timeframe - the disease of PV will not evolve into myeloid metaplasia. Spivak concedes that myelofibrosis may belong to the disease vector of PV, but he rejects bone marrow failure (and thus the concept of the marrow being spent) as the eventual outcome in the absence of cytotoxic "therapies".

 ISSN 1920-3799

8) Most PV cases that evolve into AEL and other forms of AML are caused by mutagenic therapy, whether involving ionizing radiation or alkylating drugs ("radiomimetic chemotherapy"), often administered when existing guidelines do not even call for it. Thus, most leukemogenesis in PV also appears to be iatrogenic. Writes Spivak: "acceptance of the leukemogenicity of irradiation in PV was delayed by the belief that leukemia was an inevitable feature of the disease" [170]. Spivak points out that AML sometimes coexists with the erythrocytosis phase of PV [180], and that therapy-induced (iatrogenic) acute leukemia has also been observed in patients with secondary erythrocytosis mistakenly treated with mutagenic radiation or chemotherapy [181-183].

Thus, Spivak argued that neither iron-deficiency and refractory anemias, nor evolution into bone marrow failure and acute leukemia are symptoms that necessarily belong to PV. Most are, in fact, iatrogenic artifacts, with no more value than physicians experimenting with humans under poorly controlled conditions. The essence of PV would be that it is a neoplasia or malignancy that is not "pre-leukemic", or necessarily conducive to acute leukemia.

Despite Spivak's many good points drawn out above, the iatrogenic interventions and the anecdotal nature of many of the "findings" on which the concept of a natural history of PV, even revised, has rested, the evidence today, after the discovery of the *JAK2* mutation and the interaction of JAK2 with *SOCS* genes and the IGF-I axis, suggests that PV should be seen as a variant of a set of chronic myeloid neoplasms - parallel to the chronic leukemias - whose natural history has a tendency, unrelated to radiation or chemotherapy, to develop Ph+ CML, and thus engage the auto-oncogenic vector linking progression of CML to AML [167]. In fact, in 29 different reports, PV is the most common (65%) myeloproliferative disorder preceding CML [167], and the progression takes on average 7 years to occur. Like chronic leukemias, PV and primary myelofibrosis (PMF or IMF with MMM) have a "natural' tendency to evolve towards acute leukemias. In this respect, it is most curious that Spivak invokes evidence for evolution and succession of PV clones in the same patient [184] as an argument to consider the possibility that leukemia might develop from the malignant PV clone directly. But while one study of Ph+ CML has reported coexistence of the *JAK2V617F* and *bcr-abl* mutations in both BFU-E and CFU-GM cells [185], others found no *bcr-abl* mutations in erythroid colonies, or found evidence that the two mutations belong to distinct myeloid PV/CMPD and Ph+ CML clones [167, 186]. Significantly, Pingali et al found that, while *JAK2* activation was necessary for the *bcr-abl* transformation, possibly by promoting genomic instability of myeloid stem cells, the *bcr-abl* clone was dominant and suppressed the *JAK2V617F* clone, the latter being re-expressed when the CML was treated with imanitib [167].

In order to fully understand the implications of these findings, we must point out that in

 ISSN 1920-3799

modern diagnostic and classification schemes of blood neoplasms, the term "erythroleukemia" has a very narrow meaning - it is considered to be just a subtype of acute myelogenous leukemia: acute myeloblastic leukemia (AML-M6, see below). The existence of an oncogenic progression vector has become apparent in myeloid and lymphoid leukemias (see below), presenting a progression from chronic to acute manifestations, or from cytotis to blastosis, typically across a blast crisis as seen in late CML (more on this below). If there is a comparable progression vector for erythroleukemia, it is not readily apparent.To begin with, there is no chronic erythroleukemia. The adaptive human neoplasm that exhibits an erythroid cytosis equivalent to the cytoses of chronic myeloid or lymphoid leukemias is PV. (A similar situation exists with respect to the megakaryocytic compartment and ET.) Yet, within the scope of existing clinical and research data, it is entirely possible to articulate an auto-oncogenic erythroleukemic vector where PV functions as a chronic erythroid neoplasm that progresses through a MDS-type blast crisis to acute erythroleukemia and monocytic and granulocytic forms of acute myelogenous leukemia. Conversions of PV into acute erythroleukemia have been observed in various instances [187-191], all of which Spivak fails to mention. And the already stressed overlap between CMPDs, and PV in particular, with Ph+ CML, including the frequent presence of an erythroleukemic phenotype associated with the blast crisis of CML, all suggest that underlying a substantial portion of acute myeloid leukemias there is an *auto-oncogenic erythroid vector* that promotes the *Ph+ auto-oncogenic vector* (more on this below) and eventually merges with it.

Accordingly, PV should be regarded as a neoplastic clonal disorder with an inherent potential to evolve into chronic and acute forms of myeloid leukemia, CML being the preponderant chronic form and AEL the preponderant acute form.

One can then summarize the fundamental characteristics of PV, as follows, by:

(1) a dominant erythrocytosis in the trilineal myeloproliferative clone;

(2) erythropoietic independence from the control by the physiological hormone EPO;

(3) hypersensitivity of erythroid progenitor and myeloid stem cells to IGF-I (which may be regarded as a form of dysplasia, or an anaplastic reversion to a fetal erythropoietic mechanism);

(4) non-responsiveness of erythropoiesis to suppression by hyperoxia [192]; and

(5) a single mutation in the JAK/STAT signalling pathway shared with other CMPDs, within a hot cluster region that is also hit in myeloid [167, 193] and lymphoid [194] leukemias, as well as in somatic cancers [128].

To this we may indeed add:

(6) association with distinct anemic phases as PV progresses (a correctable microcytic anemia; a pernicious anemia with megaloblastic characteristics; a refractory anemia associated with myelofibrosis; and the aggravation of all anemias upon evolution into chronic and acute leukemias);

 ISSN 1920-3799

(7) the evolution of the disease into myelofibrosis;

(8) the myelofibrosis-associated potential for further evolution of PV into marrow failure and extramedullary hemopoiesis (myeloid metaplasia) with the "spent stages of the disease" presenting not just erythropenia (leading to anemia) but a generalized myeloid cytopenia;

(9) an inherent potential to convert into chronic and acute forms of myeloid leukemia, in particular Ph+ CML, acute erythroleukemia and other forms of AML.

It is thus possible and desirable to retain the notion that PV deploys an inherent oncogenic vector that concatenates a variety of increasingly more severe neoplastic symptoms, and thus that its natural history ultimately leads to acute myeloid leukemia.The neoplastic PV vector would flow as shown in **Fig. 26**, to be contrasted with the natural history models of **Tables 7 & 8**.

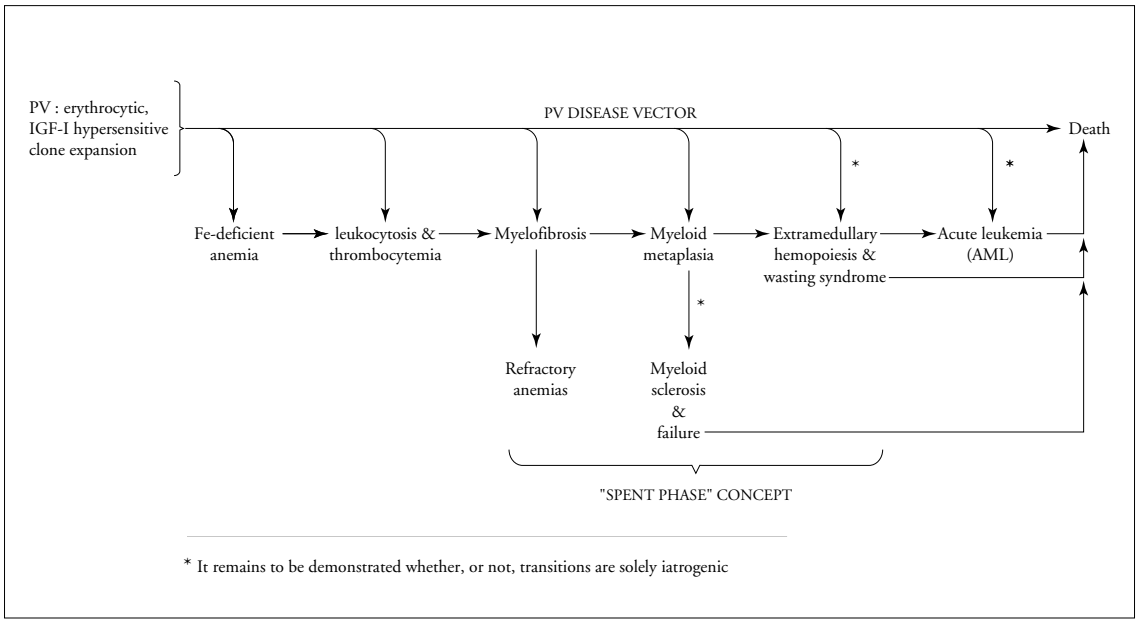

Fig. 26 - A revised auto-oncogenic PV vector. Some branchings of the vector may or may not be solely iatrogenic.

6.2. The relationship of PV and the CMPDs to MDS and acute leukemia

It is still generally held that in the polycythemias or chronic myeloid proliferative disorders the deregulation of proliferation affects only affects the size of the compartment, not cellular differentiation, whereas in myeloid leukemias both cell growth and differentiation are deregulated. But it is no longer possible to retain this distinction in order to separate hyperplasia from neoplasia, ie 'disorders of hyperproliferation" from 'neoplastic or leukemic disorders'. Rather, the so-called hyper-

 ISSN 1920-3799

plastic disorders (CMPDs) are not only malignant to the host, but already neoplastic in their own right (and, of course, every form of neoplasia involves hyperplasia), as they present more than just deregulated proliferation - they present altered differentiation, altered cellular and molecular responses in their differentiation, including altered immunophenotypes. They also present activation of a potent oncogene, in the form of the JAK2V617F mutation. The distinct myeloproliferative responses may emphasize a given hematopoietic lineage, but they are ultimately pan-myeloid, since the target of the neoplastic transformation is a myeloid stem cell.

Progression of PV always involves other affected myeloid lineages - with the megakaryocytic being the most common (50% of patients present thrombocytosis at the time of diagnosis). According to one study, 65% of PV cases present or develop some degree of granulocytosis [172]. Crossover between the CMPDs is frequent, as some patients with essential thrombocytosis develop polycythemia *vera* or idiopathic myelofibrosis [195], with dual myeloid/megakaryocytic or purely megakaryoblastic forms of AML being the eventual outcome.

Can one vectorialize a connection between the chronic myeloproliferative disorders (CMPDs) and the myeloid leukemias, as proposed above for MDS? As we said, the CMPDs include ET, PV and MMM/IMF (or PMF), all of which are clonal disorders that result from abnormal proliferation of a pluripotential myeloid stem cell, at the CFU-GEMM, or even some pre-CFU-GEMM level. Though from the beginning every CMPD is a myeloid stem cell neoplasm, each initially bears a lineage-specific emphasis which, with progression of each CMPD, tends to disappear in favor of the expression of a less differentiated, myeloid stem cell phenotype associated with blast crisis or myeloblastosis.

It is also unclear whether MMM/IMF is really a distinct clinical entity, since primary myelofibrosis with myelodysplasia - with or without extramedullary hemopoiesis - is (1) encountered in myelophtisic anemia, is (2) the main symptom of MDS - and thus, of dysplastic anemia - and is (3) likely a general response to the decomposition of the marrow environment, as encountered in PV, CML and AML/AEL. The progression of myelofibrosis with myelodysplasia to myelosclerosis and pancytopenia characteristic of MDS may be nothing other than the eventual outcome of a marrow already disordered by chronic myeloid proliferation getting depleted of the normal stem and progenitor cells that it needs to recruit, to respond to the increasingly stressed hematopoietic demand for blood cells. We should keep in mind that production of dysplastic end-point myeloid cells cannot satisfy the bodily requirement for differentiated blood cells, since the dysplastic cells are effectively dysfunctional. Thus, the combined hyperplastic-dysplastic adaptive response to the continued presence of an oncogenic stress on hematopoiesis not only fails to remove the stress, but instead aggravates it in a vicious circle. From a neo-lamarckian perspective, this could be the adaptive link underlying the myeloid metaplasia that is manifested with the progression of myelofibrosis, PV and ET, and is

 ISSN 1920-3799

associated with RARS, CMML, Ph+ CML blast crisis and AML: the increasingly more desperate requirement of the body to generate adequately differentiated myeloid cells, combined with their critical lack and marrow failure, force the body to seek extramedullary sites of hemopoiesis.

The notion of a transition from a dysplastic marrow to extramedullary hematopoiesis provocatively tallies with unpublished findings of Axelrad's group regarding the presence of circulating *stromal* progenitor cells in the peripheral blood of patients with MMM/IMF, an abnormality that seemed to differentiate MMM/IMF from the other CMPDs, which have no such cells in peripheral blood and whose abnormalities affect specific myeloid progenitors [196]. All would happen then as if, once marrow fibrosis and dysplasia set in, the body sent stromal cells in search of 'viable' extramedullary sites - which, in essence, prove to be fetal and embryonic sites. Once these sites of hematopoiesis were "revived", myeloid metaplasia would set in.

As we have seen already, all the CMPDs involve more than one hematopoietic lineage, and typically cross a MDS-like IMF/PMF stage (see **Fig. 26**) in the clinical progression of the oncogenic vector, with resulting marrow pancytopenia. It is more likely that the connection of myelofibrosis to MDS on one hand, and to the CMPDs and myeloid leukemias on the other, may be an inevitable consequence of the disruption of the normal marrow cellular structure, and one that possibly mobilizes two distinct types of stem cells. The hyperproliferation of a myeloid stem cell may be all that is needed to disrupt the hematon and trigger adaptive responses on the part of both stromal cells of hematopoietic origin (typically monocytes and T cells), and the stromal cells from connective tissue. In this scenario, increased marrow reticulin and fibrocytosis become secondary responses to the myeloid stem hyperplasia and the resulting hematon destruction. This dovetails with the notion that the early stage of IMF/PMF is not fibrotic. A clinicopathological study of 79 patients found that the prefibrotic stages of IMF/PMF presented mild to moderate refractory anemia, slight splenomegaly, hypercellular marrow with disrupted structure, granulocytosis and often ET-like thrombocytosis, in the absence of any signs of marrow fibrosis [197]. It also suggested that the prefibrotic stage should be taken into account in the diagnosis of PMF/IMF. Without knowing whether splenic myeloid metaplasia was active in the patients studied, the slight splenomegaly characteristic of the prefibrotic stage of IMF/PMF may already betray an ongoing process of precocious degradation of RBCs, and even one involving deregulated auto-immune responses.

In the same scenario, the evolution of myelofibrosis to myeloid metaplasia would then involve either marrow export of the stromal cells of hematopoietic origin to the spleen, or their activation in the spleen and subsequent release to the circulation. There would then be a reason to link IMF/PMF to MMM, and to speak of a myelodysplastic syndrome (MDS) associated with myeloid neoplasms, whether CMPDs or leukemias. The dysplastic, fibrotic and sclerotic phases of marrow would be part

 ISSN 1920-3799

of an integral process whose outcome - jointly shared by the CMPDs and myeloid leukemias - was marrow pancytopenia and myeloid metaplasia (eg MMM).

Finally, it is suggested that the CMPDs and MDS have a relation of progression towards acute myeloid leukemia parallel to that of CML. Pre-blast crisis Ph- CML may exhibit a JAK2 mutation (*TEL*|*JAK2* fusion, see **Table 14**). The parallel footing of CMPDs, MDS and CML is suggested irrespective of the promotion of Ph+ CML by the JAK2 mutation shared by all three CMPDs, since this instance concerns only the progression of the erythroleukemic auto-oncogenic vector towards the Ph+ panmyeloid auto-oncogenic vector during blast crisis. As in the progression of the CMPDs, MMM is encountered in CML blast crisis, and typically this coexists with the appearance of erythroblasts and nucleated red blood cells in peripheral blood, forming a picture analogous to that encountered in AEL. Thus, like the vectors of the natural history of PV and MDS, CML also develops into AEL. To summarize - chronic myeloid neoplasms should therefore include not only the CMLs, but also the CMPDs and MDS, all of which share a comparable potential to evolve into forms of AML.

 ISSN 1920-3799

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153. For a peer-reviewed paper published by the New York Academy of Sciences, this constitutes another example of the glaring basic failures of peer-review, which are more accentuated today than ever before in the history of institutional science. It suffices for a paper to have a large number of authors associated with "reputed institutions", as is the case with Steidl et al [153], for the science to to be less scrutinized. One can only wonder about human guinea pigs being subject to such toxic concentrations of ATRA under the guise of treatment - and be amazed as to how grants are allocated and "potential cures" are marketed on the basis of such studies.

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