the initial haplotypic pattern on which HbO-Arab evolved (haplotype Greek VI) can be attributed. A *de novo* mutation event leading to the formation of HbO-Arab was superimposed on that certain haplotype, whose frequency in the meanwhile increased due to genetic drift. Using the coalescent method to compute TMRCA it appears that this happened 0.252±0.216 coalescent units ago. A second recombination event, between the 3' and 5' subhaplotypes of the Greek haplotype VIc and the universal haplotype I followed before 0.032±0.049 coalescent units and accounts for the minority of HbO-Arab-carrying Pomak chromosomes that display a variety of the universal haplotype I (Id). As a coalescent unit (measured in years) represents the sum $2^*N^*t_g$ (where N is the population size and t_g is the mean years passed per generation), the approximate time elapsed since the two above-mentioned events is 2000 and 250 years, respectively (for N=200, which is a reasonable estimate for isolated populations, and t_g=20) (Figure 1).

Summing up, we found that 42% of the normal Pomak chromosomes display two unusual *HBB* cluster haplotypes, one of which is also strongly associated with the HbO-Arab mutation, and that 33% of normal Pomak chromosomes carry the CCTCT *HBB* framework, with which all HbO-Arab chromosomes are linked. These results suggest that Pomaks constitute a population group characterized by a high genetic drift that can be attributed to historically confirmed physical isolation and intense inbreeding. This environment might have given rise to novel haplotypes, as products of intra-*HBB* cluster recombination events, in one of which the HbO-Arab mutation emerged and increased in frequency. As the HbO-Arab related haplotype and framework is widely dispersed among normal Pomak chromosomes and not in any other known population,[2] the HbO-Arab mutation might have emerged among Pomaks. Heterozygosity for HbO-Arab may be beneficial, which would further ensure the survival of the mutation. Pomaks might have spread HbO-Arab throughout the Mediterranean basin. Minor HbO-Arab related haplotype variations reported[9,10] could be attributed to local recombination events. Finally, the uniformity of the presence of the CCTCT framework and the close association with the Greek VIa haplotype in all our HbO-Arab cases as well as all others already reported in the literature – with only minor variations – indicate a single origin of the mutation.

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Red Cell Disorders

Phosphoglycerate mutase BB isoenzyme deficiency in a patient with non-spherocytic anemia: familial and metabolic studies

We previously reported the first case of red blood cell phosphoglycerate mutase (PGAM) isozyme BB deficiency due to the homozygous point mutation cDNA 690G→A, which causes a substitution of methionine 230 by isoleucine. In the present work we analyzed the changes in glycolytic intermediates caused by this mutation. With the exception of hexose phosphates, all other intermediates were decreased. In contrast, lactate levels were increased. The methionine 230 isoleucine change did not alter the mutated PGAM levels.

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Phosphoglycerate mutase (PGAM, EC 5.4.2.1) is a glycolytic enzyme that catalyses the interconversion of 2-phosphoglycerate (2PGA) and 3-phosphoglycerate (3PGA), with 2,3-bisphosphoglycerate (2,3-BPG) being required, in mammals, as a co-factor. In addition to acting as a PGAM cofactor, 2,3-BPG in red blood cells (RBC) acts as an allosteric modulator of the affinity of hemoglobin for oxygen and is present at much higher concentrations than in other cell types. In mammals, the enzyme is present in three isozymes which result from the homodimeric and heterodimeric combinations of two different subunits, M and B, coded by two different genes, although the gene coding subunit B is unknown. Only

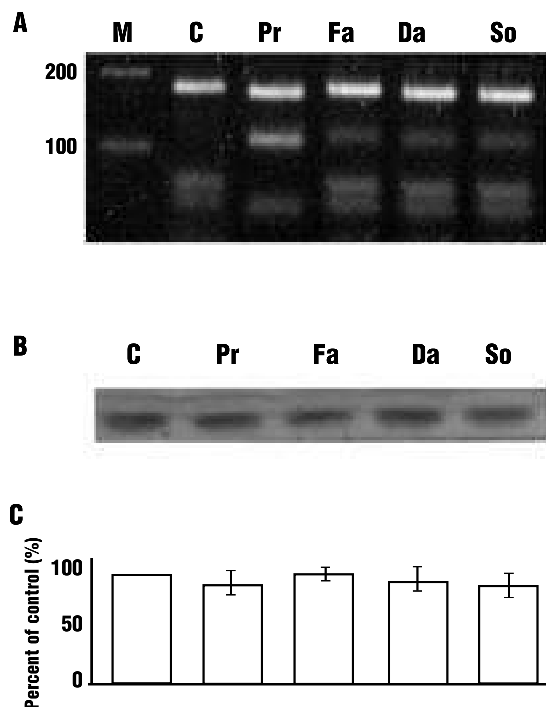

Figure 1. A. *Nla*III restriction analysis. Control shows the 72 bp normal band which generates a 111 bp in the proband. All family samples show a heterozygous band pattern with the normal 72 and the 111 bp bands. B. Western-blot analysis. 100 µg of protein were loaded in each lane. C. Quantitative densitometry analysis of healthy controls (n=12) and the proband and her family (each analysis repeated 5 times). Bars show the standard deviation. M: molecular weight marker; C: control; Pr: proband; Fa: father; Da: daughter; So: son.

the homodimer BB is present in RBC.[1] Several cases of deficiency of PGAM-MM isozyme, only present in muscle and mature sperm cells, have been described,[2] although only three different mutations have been identified.[3] In contrast, only one PGAM-BB deficiency has been reported. The patient, a 34-year old woman, was homozygous for the Met230Ile substitution and had normocytic anemia and hereditary spherocytosis. Detailed clinical traits, as well as restriction, genetic and conformational analyses of the patient, were recently reported.[4]

Western blot analyses were performed as previously reported;[5] bands were quantified using the *Quantity One* program, version 4.1.1 and the area of the control band was used as a normalizing parameter. Glycolytic intermediates were measured as described by Minakami *et al.*[6] 2,3-BPG analyses were performed using the SIGMA kit following the manufacturer´s instructions. RNA was isolated from white cells using the Tripure™ isolation reagent method (Roche Applied Science). Reverse transcription polymerase chain reaction (RT-PCR) amplification was performed according to the manufacturer's instructions using the Retrotools cDNA/DNA Polymerase Kit (Biotools) two-step protocol. Mutation analyses were performed with the restriction enzyme *Nla*III in 20 µL of PCR product.[4]

The cDNA 690G→A mutation was searched for in the patient, her sons and her father. Unfortunately, the mother had died. Unlike the patient, who presented the mutation in homozygosis, her family are heterozygote carriers of the mutation (Figure 1A) and are healthy. Western blot analyses showed no significant differences between controls, the patient and her family; thus, changes in concentration of the mutated enzyme can be ruled out (Figure 1, B and C). PGAM activity was reduced by 50% as a result of the mutation. The concentrations of all other glycolytic, pentose pathway and glutathione metabolism enzymes were in the normal ranges (*data not shown*). The analysis of the patient's RBC metabolites is depicted in Table 1. As can be seen, all intermediates from fructose diphosphate to pyruvate, decreased by approximately 50%, while glucose-6-phosphate and fructose-6-phosphate were normal. It is noteworthy that lactate levels increased to 150% of those present in the controls.

Table 1. Metabolite analysis of the PGAM-BB-deficient patient. All values are expressed as nmol/mL RBC, with the exception of lactate and pyruvate which are indicated as nmol/mL blood.

	Control (n=7)	Proband
G6P	49±15	35±1
F6P	20±8	19±1
FDP	49±15	14
GAP	19±6	7±2
DHAP	40±9	14±2
2PGA	26±10	10
3PGA	48±8	18±1
2,3-BPG	3183±114	1912 ± 44
PEP	33±10	17±6
PYR	50±5	8±1
Lactate	856±115	1286±41
ATP	1748±251	1590±32
ADP	203±34	175±1
AMP	37±8	36±2
NAD^+	55±7	79±19
P-gluconate	240±140	121±34

The most usual consequence of glycolytic enzyme deficits is a decrease in concentrations of metabolites located immediately after the deficient enzyme. However, this did not occur in the present case since not only did the level of 3-PGA, a PGAM product, decrease but so too did levels of all glycolytic intermediates from fructose diphosphate to pyruvate. In contrast, lactate levels were increased. These unusual alterations (a decrease in practically all intermediates), could only be explained by significant changes in RBC features. The most plausible cause of all these changes is a decrease in intracellular pH due to the increase in lactate concentration. A decrease in pH is known to inhibit phosphofructokinase;[7] this slows the glycolytic rate and all glycolytic metabolite intermediates from PFK, including lactate, also decrease. However, in this patient, lactate was increased instead of decreased. This RBC lactate accumulation is abnormal and, although the cause of this situation is not clear, it can only be explained by an alteration in lactate export. Consequently, our patient showed two different abnormalities, loss of PGAM activity and lactate accumulation, probably due to two different causes: the former due to PGAM 690G→A mutation and the latter, altered lactate permeability, maybe the results of reduced passive efflux rates for this metabolite. Therefore, further studies on protein membrane alterations and other possible abnormalities in this patient have been started.

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Chronic Myeloproliferative Disorders

Clonality analysis by *HUMARA* assay in Spanish females with essential thrombocythemia and polycythemia vera

Analysis of the human androgen receptor gene (*HUMARA*) allows clonality to be assessed in essential thrombocythemia (ET) and polycythemia vera (PV). We studied clonality in 44 patients with ET, 18 with PV and in 64 healthy controls. The X-chromosome inactivation pattern was analyzed by HUMARA-polymerase chain reaction on DNA from purified granulocytes, T lymphocytes and the CD3⁻ fraction of mononuclear cells.

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Essential thrombocythemia (ET) and polycythemia vera (PV) are clonal myeloproliferative disorders without consistent karyotypic and molecular markers. *HUMARA* analysis, an assay based on X-chromosome inactivation patterns (XCIP), allows clonality to be assessed in ET and PV. There is controversy over the percentage of clonal hematopoiesis in ET and little is known about this feature in PV patients.

The present study was conducted in a cohort of Spanish ET and PV patients to determine: (i) the percentage of clonality in these disorders; (ii) differences in frequencies of clonality depending on whether granulocytes or $CD3^-$ mononuclear cells are taken as the pathologic tissues and, (iii) the relationship between clonality status and the appearance of thrombotic and hemorrhagic complications. One hundred and twenty-six people were included in this study; 44 had ET, 18 had PV and 64 were healthy, age-matched controls. The diagnoses of ET and PV were established according to the PVSG criteria.

Mononuclear cells and granulocytes were isolated from peripheral blood. $CD3^+$ lymphocytes were then separated from the $CD3^-$ fraction by magnetic beads. Genomic DNA was extracted from each fraction. The *HUMARA* assay was performed according to normal procedures in our laboratory. Clonality was calculated after correcting for the degree of lyonization in $CD3^+$ cells. Cells were considered to be clonally derived when the corrected allele ratio was <0.25. The sensitivity and specificity of the *HUMARA* assay in our study were 39% and 94%, respectively. The rate of heterozygosity of the *HUMARA* gene in our Spanish women was 88%. Table 1 shows the clinical data and *HUMARA* results from the ET and PV patients and the controls. We found significant differences in clonality results between controls and patients (p<0.001). Clonality in normal controls has also been reported by other authors[1-9] (Table 2). Champion *et al*. suggested that this phenomenon could be more frequent in elderly women, probably due to constitutive skewing, age-related acquired skewing or genetic factors leading to selective pressure.[2] This might suggest that imbalanced myeloid XCIP in the presence of balanced T cell XCIP in elderly patients (>65 years) should not be certainly interpreted as clonal hematopoiesis.

A wide range of positivity for clonality has been reported in ET patients (18.7-68%).[1-9] These differences might be attributed to methodology, number of studied patients and criteria used to define a clonal pattern (allele ratios or the equation of Asimakopoulos *et al.*[10]) (Table 2). There are two studies reporting on clonality in PV. In one, 26/41 patients (8 ET, 30 PV and 3 primary myelofibrosis) had clonal proliferation,[2] and in the other, in which 31 PV patients were studied, hematopoiesis was clonal in 16/22.[6]

Clonality analysis in ET and PV has mainly been performed using granulocytes and platelets as target cells. A major limitation of platelets is that the study has to be performed on mRNA instead of DNA, using polymorphisms that are less heterozygous than the *HUMARA* gene. For this reason, we investigated not only granulocytes but also $CD3^-$ mononuclear cells as these have been suggested to share a common abnormal hematopoietic precursor cell. Our data show that when $CD3^-$ cells were tested instead of granulocytes, clonality was not detected in 13 patients and therefore a substantial number of clonal results could be missed (p<0.001) (Table 1).

Although no significant correlation was found between clonality status and the patients' age, there was a statistical trend to more clonal cases among elderly (>65 year) women than among younger women (p=0.180). This trend, not significant in our study, had been found to be statistically significant by other authors.[4,9] When skewed cases were considered, there was statistical trend of more skewed cases in elderly women (p=0.075).

It is known that the prevalence of thrombotic complications is strongly influenced by a patient's age and duration of follow-up. In addition, some authors observed that thrombosis occurred more frequently in clonal than in polyclonal ET patients.[5,7,9] In contrast, El-Kassar *et al.*[3]

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