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Catherwood, M. A., Graham, A., Cuthbert, R. J. G., Garrec, C., Gardie, B., Girodon, F., ... McMullin, M. F. (2018). Absence of CALR Mutations in Idiopathic Erythrocytosis Patients with Low Serum Erythropoietin Levels. Acta haematologica, 139(4), 217-219. DOI: 10.1159/000489006

Published in:
Acta haematologica

Document Version:
Peer reviewed version

Queen's University Belfast - Research Portal:
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Absence of *CALR* mutations in idiopathic erythrocytosis patients with low serum erythropoietin levels

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Key words: Idiopathic erythrocytosis, JAK2, calreticulin, erythropoietin

Short Title: *CALR* mutations in idiopathic erythrocytosis

Idiopathic erythrocytosis (IE) is a heterogeneous collection of rare haematologic disorders which can be either sporadic or familial in origin (McMullin *et al*, 2005). An erythrocytosis occurs when there is an increased red cell mass and an elevated haematocrit. IE is distinct from polycythaemia vera (PV) as the red cell hyperplasia present in IE is not accompanied by elevations in the megakaryocytic or granulocytic lineages and PV is characterized by low serum erythropoietin (Epo) levels, whereas IE is associated with a wide range of Epo levels. IE can be divided into two main groups. The first group consists of those with a low EPO level suggesting an unidentified molecular defect possibly involving components of the Epo signal transduction pathway (de la Chapelle *et al*, 1993). The second group are those with inappropriately normal or raised EPO level which suggests a secondary unidentified cause possibly affecting the oxygen sensing pathway. These patients are rarely observed in clinical practice, and little is known regarding their clinical characteristics, natural history and best management.

Recently, novel frameshift mutations in exon 9 of the calreticulin (*CALR*) gene were found in patients with *JAK2* or *MPL* unmutated primary myelofibrosis and essential thrombocythemia (ET) (Klampfl *et al*, 2013; Nangalia *et al*, 2013). Even though mutations of *CALR* have been reported in significant numbers in MPN it was initially thought that *CALR* mutations did not occur in PV. However in 2014 two *JAK2* negative PV patients were described that harboured *CALR* mutations (52 bp deletion) (Broseus *et al*, 2014) with further cases described in other studies (Chauveau *et al*, 2017).

The aims of this study were to assess the prevalence of *CALR* mutations in (i) a well characterized cohort of IE patients with low serum erythropoietin levels and (ii) a broad cohort of query myeloproliferative neoplasm (MPN) cases referred for molecular genetic investigation.

Initially we studied 79 patients with IE. Thirty eight were enrolled from a UK cohort and 41 from a French cohort.
Ethical approval was granted by the local review committees and informed consent was collected according to the Helsinki Declaration.
All the patients had low to normal serum EPO level (Table 1) and none of them carried *JAK*2V617F or *JAK*2 exon 12 mutations as determined by allele specific PCR. Clinical and laboratory details of the patients are shown in Table 1. The procedures followed were in accordance with the Declaration of Helsinki and all patients gave informed written consent. *CALR* exon 9 mutations were screened by fragment length analysis according to Klampfl *et al*, 2013.

The patients were mostly males in both the groups with a higher frequency in the French cohort. Patients in both cohorts had similar characteristics (Table 1). No *CALR* mutations were detected in any of the 79 IE patients included in this study with low to normal EPO levels.

To further explore the possibility of *CALR* mutations in PV we audited samples referred for MPN panel analysis at the Wessex Regional Genetics Laboratory (WRGL) in Salisbury (Jones *et al*, 2015). Since October 2016 all query MPN referrals to the WRGL have been tested using an amplicon-based next generation sequencing approach for *JAK2* V617F, *JAK2* exon 12, *MPL* exon 10 and *CALR* exon 9 mutations irrespective of their phenotype. Of 2306 referrals, 137 (5.9%) tested positive for *CALR* +1 frameshift mutations. None of the 659 referrals that specified an erythroid phenotype PV, ?PV, high haematocrit or high haemoglobin) tested positive for *CALR* mutations.

In previous studies 4 *CALR* mutations have been identified in so called *JAK2* negative PV in patients more accurately described as *JAK2* negative unexplained erythrocytosis. In the first study only 2 patients were studied (Broseus *et al*, 2014). Both cases were *JAK2* negative PV and the *CALR* mutation was a 52bp deletion, commonly termed type 1 mutation. Of note, both patients had thrombocytosis in addition to erythrocytosis. In the second study a large cohort of 578 patients were examined (Chauveau *et al*, 2017). Of the two patients with *CALR* mutations one was subsequently considered to have ET with slightly increased red cell mass and the other considered to have idiopathic erythrocytosis. Both cases harboured the 52bp deletion. Of interest, in the patient with idiopathic erythrocytosis the *CALR* mutation was detected with a low allele burden of approximately 5%.

The underlying defect in cases with unexplained erythrocytosis provides a clinical conundrum. Our findings indicate that *CALR* mutations are absent in a cohort of 79 patients with IE and also in a large, broad real-life cohort of patients with an erythroid phenotype referred for MPN panel testing. We therefore consider that the screening of *CALR* mutations in this situation is not warranted as a standalone investigation.

"Disclosure Statement"
The authors declare no conflicts of interest.

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Authorship

AG analysed the data, wrote the paper and performed the molecular analysis. MC, AG, SL and BG performed the molecular analysis. RJGC contributed clinical data and followed the patients. MFM, NCPC and FG designed the study, recruited patients and critically reviewed the manuscript. All authors have read and approved the final version.

Table 1. Patients characteristics.

UK

	Mean	Range/percentage
Sex, female:male	11:27	29% female
Age	54	27-75
Haemoglobin level, g/L	198	169-249
Hematocrit, %	57	49-79
Platelet count, 10^9/L	220	119-467
White blood cells, 10^9/L	8.3	4.2-15.6
Erythropoietin level, mIU/mL	5.8	1-9.9

FRENCH

	Mean/ratio	Range/percentage
Sex, female:male	3:38	5% female
Age	48	13-79
Haemoglobin level, g/L	186	161-230
Hematocrit, %	52.7	46-66
Platelet count, 10^9/L	215	139-350
White blood cells, 10^9/L	7.7	5.34-9.14
Erythropoietin level, mIU/mL	11.35	1.7-30

BOTH

	Mean/ratio	Range/percentage
Sex, female:male	14:65	18% female
Age	51	13-79
Haemoglobin level, g/L	192	161-249
Hematocrit, %	54	46-79
Platelet count, 10^9/L	218.5	119-467
White blood cells, 10^9/L	8	4.2-15.6
Erythropoietin level, mIU/mL	8.6	1.7-30