Central Manchester University Hospitals NHS
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Clinical details are vital for the effective application of a targeted *CALR* mutation service for *JAK2* negative referrals

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Background

- A significant proportion of Myeloproliferative Neoplasm (MPN) cases, excluding CML, have no visible chromosome abnormality. However, acquired molecular markers can provide additional prognostic information.
- The somatic *JAK2* V617F mutation is present in a majority (95%) of polycythaemia Vera (PV) patients, but only in 50-60% of patients with Essential Thrombocythaemia (ET) and Idiopathic Myelofibrosis (IMF). In a small percentage of *JAK2* V617F negative PV patients, other mutations can occur within Exon 12 of the *JAK2* gene.
- Mutations in the thrombopoietin receptor gene *(MPL)* have been identified in 5-10% of patients with *JAK2* V617F negative ET and IMF.
- Detection of these mutations can be used to distinguish between a true MPN and a reactive disorder, improving diagnosis. However the diagnosis of ET and IMF in patients without these molecular markers is more challenging.
- Recently, somatic frameshift mutations (insertions or deletions) in exon 9 of the calreticulin gene *(CALR)* were found to be present in a majority (70-85%) of ET and IMF patients who were mostly lacking *JAK2* or *MPL* mutations and is the second most frequent somatic mutation after *JAK2* V617F in these patients.

Mutation	MPN		
	PV	ET	IMF
JAK2 V617F	95-98%	50-60%	50-60%
JAK2 Exon 12	Up to 5%	0%	0%
MPL	0%	5-10%	5-10%

Table 1: The incidence of the common mutations present in the classical MPNs.

CALR

- The *CALR* gene encodes calreticulin, an endoplasmic reticulum (ER) luminal Ca^{2+} -binding chaperone protein with a critical role in glycoprotein folding and several other cellular functions both inside and outside the ER.
- All *CALR* mutations reported to date lead to an altered reading frame and result in a mutant protein with a novel C-terminal.
- The most common mutations are a 52-bp deletion (c.1092_1143del, L367fc*46) and a 5-bp insertion (c.1154_1155insTTGCC, K385fc*47), comprising approximately 85% of *CALR* mutations in MPN.
- *CALR* mutations have been found in the haematopoietic stem and progenitor cells in MPN patients and may activate the STAT5 signalling pathway.
- ET and IMF patients with *CALR* mutations have lower haemoglobin, higher platelet counts, lower risk of thrombosis and a better overall survival than patients with mutated *JAK2*.

Aim

- Due to the high prevalence of *CALR* mutations in *JAK2*-negative ET and IMF patients it has been suggested that the BCSH diagnostic criteria for diagnosis of thrombocytosis be modified to include *CALR* testing, along with *JAK2* and *MPL*, in the further investigation of persistent unexplained thombocytosis.
- The current study sought to determine where additional *CALR* testing of *JAK2* V617F negative patients was relevant and establish the frequency of *CALR* mutations found in a selected cohort of patients.

Methods

Patients

- The clinical details provided for all year 2014 referrals to the Molecular Oncology service at MRI were reviewed to determine whether *CALR* testing was required. Those selected for the patient cohort included all referrals that were *JAK2* V617F negative with clinical features not associated with PV and those where no clinical information had been provided.
- 638 patients were identified from this process (Fig. 1) and were screened retrospectively for the presence of *CALR* exon 9 indel mutations using a fragment analysis assay with sensitivity for the mutant allele burden of 5%.

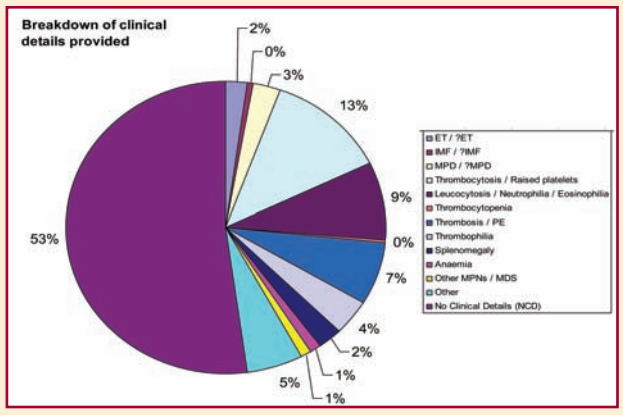

Figure 1. Breakdown of clinical details provided for referrals that were included in the 638 patient cohort where *CALR* was performed.

CALR fragment analysis assay

- PCR was carried out on patient genomic DNA using a forward (fluorescently labelled) and reverse primer. PCR products were analysed by capillary gel electrophoresis on an ABI 3130xl genetic analyser followed by fragment analysis on GeneMapper Software v4.0 (Applied Biosystems) Fig. 2. All samples with an additional peak to the *CALR*-WT (wild type) were further analysed by Sanger sequencing.

Figure 2. PCR fragment analysis of *CALR* exon 9 in three patients. Patient A is negative for a *CALR* indel mutation indicated by presence of the WT peak at 298-bp alone. Patient B is positive for a *CALR* 52-bp deletion indicated by presence of a mutant peak at 246-bp in addition to the WT peak. Patient C is positive for a *CALR* 5-bp insertion indicated by presence of a mutant peak at 303-bp in addition to the WT peak.

Results

- The *CALR* mutation profile detected in the patient cohort is given in Table 2.
- 6% (36/638) patients were found to be positive for a *CALR* indel mutation, with the two common mutations (52-bp deletion and 5-bp insertion) most frequently detected.
- 79% of patients referred with suspected ET were positive for *CALR* mutations (11/14). Detection was 33% (1/3) and 6% (1/18) in suspected IMF and MPD referrals respectively. 52% of the selected referrals had no clinical details provided and of these, only 4% (13/321) were positive for *CALR* mutations (Fig. 3).

CALR indel mutation types detected	Frequency
52-bp deletion	20 (56%)
5-bp insertion	8 (22%)
14-bp deletion	2 (6%)
19-bp deletion	2 (6%)
34-bp deletion	2 (6%)
46-bp deletion	1 (3%)
10-bp deletion	1 (3%)
Total	36

Table 2. Frequency of *CALR* indel mutations detected in selected cohort of 638 *JAK2* V617F negative patients.

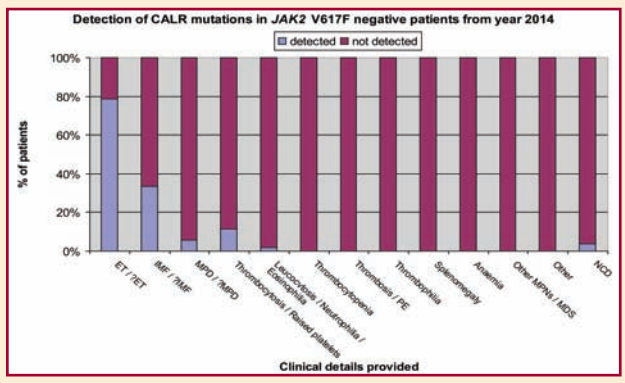

Figure 3. Detection (%) of *CALR* indel mutations within each clinical detail category.

Summary

- Thrombocytosis/raised platelets and leucocytosis were the most frequently stated clinical details.
- Clinical features associated with ET were more likely to be *CALR* positive than those which were not.
- 71% (12/17) of referrals suspecting ET and IMF were positive for a *CALR* mutation which is consistent with the expected detection rate of 70-85% in these patients.
- Overall *CALR* indel mutations were not detected in 94% of the selected referrals, reflecting the lack of clinical information supplied.

Conclusion

- The study demonstrated that, with the exception of referrals stating ET, there is a need for inclusion of specific referral information to allow resource efficient molecular diagnostic screening in relevant patients.
- In accordance with the proposed modification of the BCSH diagnostic criteria for diagnosis of thrombocytosis, such an approach has been utilised as per the suggested molecular diagnostic algorithm in Fig.4.
- In light of our observations, the Molecular Oncology service at MRI now offers a *CALR* mutation screening service which is currently clinician directed and not based on automatic reflex testing following a negative *JAK2* V617F mutation screen.

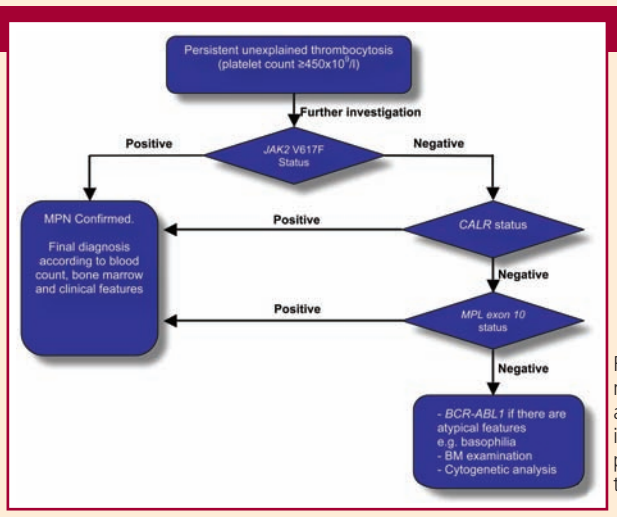

Figure 4. Suggested molecular diagnostic algorithm for investigation of persistent thrombocytosis

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