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Critically Appraised Topic

Mutation analysis of *JAK2* exon 12 and *MPL* exon 10 in myeloproliferative disorders

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CLINICAL BOTTOM LINE

Molecular diagnosis has become an important part of the work-up for BCR-ABL negative myeloproliferative disorders (MPN), such as polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). Practically all PV patients and more than half of ET and PMF patients carry a mutation in hotspot region V617F of the Janus kinase 2 (*JAK2)* gene. In 2007, Scott et al. discovered *JAK2* exon 12 variants in a significant amount of V617F-wildtype PV cases.[1] In ET and PMF, calreticulin (*CALR*) and myeloproliferative leukemia virus oncogene (*MPL*) were identified as two additional important driver genes.
UZ Leuven has a protocol in place to screen for *JAK2*, *CALR* and *MPL* mutations depending on the patient's clinical presentation. In case of V617F-negativity and suspicion of PV, PCR and Sanger sequencing for *JAK2* exon 12 is performed. For V617F-negative patients with PMF or ET, *CALR* and *MPL* are excluded by NGS, or PCR and NGS respectively.
Since Sanger sequencing cannot detect variants with low mutated allele burden and NGS for ET is not yet reimbursed, we decided to compare our current workflow to standard practice guidelines. Similar step-by-step protocols for MPN genetic testing were found. Many papers however applied more sensitive *JAK2*/*MPL* detection methods. We therefore set up an allele specific PCR protocol to test for *JAK2* exon 12 and *MPL* exon 10 mutations Here we show that this approach has a high sensitivity and may reduce the need for expensive NGS.

CLINICAL/DIAGNOSTIC SCENARIO

Blood and bone marrow samples from patients suspected of having a myeloproliferative disorder (MPN) such as polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), can be genetically evaluated by the center of human genetics (CME) at the University Hospitals Leuven (UZ Leuven). Specific mutations are investigated, according to their prevalence and clinical significance for the disease. In this way, the discovery of the V617F-mutation in the Janus kinase 2 (*JAK2*) gene has proven a very useful diagnostic tool, since more than 95% of PV patients and more than half of ET and PMF cases carry this precise genetic alteration.[2-4] The *JAK2* (V617F) mutation is a somatic gain-of-function mutation involving exon 14 of *JAK2*, which encodes a part of the JH2 auto-inhibitory domain of the JAK2-kinase. The mutation results in a constitutively active kinase which in turn causes (hyper)activation of the Signal Transducer and Activator of Transcription proteins (STAT).[3,5]
Besides the predominant *JAK2* (V617F) mutation, many other genetic alterations that contribute to MPN have been described.[2,6] In PV for example, a similar upregulation of the JAK-STAT pathway can be seen due to mutations affecting *JAK2* exon 12.[1,7] In ET and PMF however, *JAK2* exon 12 mutations have not yet been described. These patients more often experience mutations in calreticulin (*CALR*) (30% of PMF/ET cases) or suffer alterations in exon 10 of the myeloproliferative leukemia virus oncogene *MPL* (3-8%).[8-12] Another 12% of cases are triple-negative and harbor no mutations in *JAK2*, *CALR* nor *MPL*.[2,5,13,14]

Diagnostic testing for MPN at the CME involves a step-by-step process. For PV patients a workflow is installed which requires first determination of the presence of the *JAK2* V617F mutant by digital droplet PCR (ddPCR); a technique that allows multiple PCR reactions occurring in parallel in tiny droplets. Sensitivity is high, with 0.5% of mutant alleles detectable. In case of V617F negativity and strong clinical suspicion of PV, a second analysis can be performed, using PCR and Sanger sequencing, to look for mutations affecting *JAK2* exon 12. An important limitation of this Sanger sequencing approach however is the detection threshold of 10 to 20%, which does not allow low burden alterations to be identified.
For patients with PMF, samples are analysed by next-generation sequencing (NGS) using the TruSight Myeloid panel (Ilumina®), that contains primers for hotspot regions in 54 genes, including *JAK2*, *CALR* and *MPL*. The sequencing cost is covered by the NGS convention (RIZIV). In case of (suspected) ET however, NGS is performed to determine the presence of *MPL* exon 10 alterations only after *JAK2* V617F and *CALR* mutations have been excluded. With only two tests reimbursed for ET, both the ddPCR for *JAK2* V617F and the standard PCR for *CALR*

are covered. NGS for *MPL* however is not included, making *MPL* mutation analysis expensive and loss-making for the hospital.

We therefore decided to reconsider the current workflow for *JAK2* exon 12 and *MPL* exon 10 mutation analysis by comparing it to methods described in literature and to investigate the cost-benefit ratio for an alternative allele-specific (AS) PCR approach.

QUESTION(S)

1) *Are there guidelines available for genetic testing in case of (suspected) MPN and what are their recommendations for JAK2 exon 12 and MPL exon 10 evaluation?*
2) *What is the current status of JAK2 exon 12 and MPL exon 10 mutation analysis in UZ Leuven and how does our workflow compare to literature and other hospitals?*
3) *Is allele-specific PCR a valuable option for JAK2 exon 12 and/or MPL exon 10 mutation analysis?*

SEARCH TERMS

1) *MeSH Database (PubMed): MeSH term:* "myeloproliferative disorders", "polycythemia vera", "essential thrombocytosis", "primary myelofibrosis", "high-throughput nucleotide sequencing", "polymerase chain reaction", "DNA mutational analysis", "INDEL mutation", "MPL protein, human", "Janus Kinase 2"

2) *Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), National Guideline Clearinghouse (http://www.ngc.org/), Institute for Clinical Systems Improvement (http://www.icsi.org), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane, Health Technology Assessment Database (http://www.york.ac.uk/inst/crd/htahp.htm):* "myeloproliferative disorders" "polycythemia vera" "essential thrombocytosis" "primary myelofibrosis" "DNA mutational analysis" "MPL" "JAK2"

RELEVANT EVIDENCE/REFERENCES

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f) Posters, "grey literature", presentations

Not applicable

APPRAISAL

1) Are there guidelines available for genetic testing in case of (suspected) MPN and what are their recommendations for JAK2 exon 12 and MPL exon 10 evaluation?

Several guidelines exist concerning the diagnosis of MPN of which some specifically focus on the molecular diagnosis of PV, ET and/or PMF.[15-18] Two out of four reviewed guidelines already incorporated the in 2016 revised WHO standard for MPN diagnosis.[15,16] An extended overview of the current WHO-criteria for PV, ET and PMF is listed in Appendix I.
Briefly, I would like to mention that PV is a myeloproliferative disorder characterized by an abnormal proliferation of the erythroid cell line, which results in increased hemoglobin and hematocrit levels combined with subnormal erythropoietin levels. The bone marrow of PV patients is usually hypercellular, mostly due to increased numbers of red blood cell precursors and megakaryocytes. Where PV is relatively easily distinguished from other MPNs, PMF and ET share highly similar morphological features. Especially in the early fibrotic stage of PMF, differential diagnosis with ET (but also other MPNs) can be tricky. In case of PMF, proliferation of predominantly abnormal megakaryocytes causes reactive deposition of reticulin and collagen fibers in the bone marrow. Eventually, massive fibrosis necessitates extramedullary hematopoiesis resulting in prominent spleen enlargement and tear-drop shaped red blood cells on blood smears.
Patients with ET usually present with sustained thrombocytosis (> 450 $x10^9$/L) and an increase in (very) large, mature megakaryocytes in the bone marrow aspirate and/or biopsy. Dysplasia is relatively limited in ET. The occurrence of highly atypical megakaryocytes or significant fibrosis should therefore suggest the presence of PMF, rather than ET.[2]

Since virtually all PV patients carry the *JAK2* V617F-mutation in addition to 50 to 60% of ET and PMF patients, *JAK2* exon 14 evaluation is an important criterion to strengthen the diagnosis of MPN.[2,17] All revised guidelines therefore propose a gradual screen for MPN suspected patients, starting with *JAK2* (V617F) determination.[15-17] Ideally, a sensitive method is applied for V617F identification since low variant allele frequencies (VAF) have been described.[4,19-21] Methods with a sufficiently high analytical sensitivity (at least 1%, but preferentially 0.1%) include allele specific PCR (AS-PCR) and ddPCR.[17,19,22]
In 2007, Scott et al. demonstrated that *JAK2* exon 12 alterations are present in about 3% of PV patients.[1,23] For patients with a strong suspicion of PV (isolated erythrocytosis, subnormal erythropoietin levels) and a wildtype hotspot region, further evaluation of exon 12 variants is strongly advised.[15-18] Several distinct mutations and copy number alterations (insertions-deletions or indels) in exon 12 have been described.[17] Due to this diversity, PCR and Sanger sequencing are frequently used techniques for *JAK2* exon 12 analysis. Unfortunately, lower VAFs (<15%) are even more common for exon 12 alterations than for the V617F variant.[18] To avoid equivocal results with Sanger sequencing, Gong et al. advise the use of more sensitive techniques including AS-(q)PCR, clamped PCR followed by nucleotide sequencing, and melting curve analysis.[17] AS-PCR however is limited by the number of primers you add to detect distinct point mutations and copy number alterations.[17,18]
NGS could also be appropriate in case deep sequencing is applied. Although NGS is more expensive, it allows additional exons (and genes) to be screened in parallel.[22,24,25] Some rare mutations outside exon 12 and 14 have indeed been identified (Appendix II).[17,26,27] An overview of the different available techniques for JAK2 mutation analysis and their sensitivity threshold is provided in Appendix III.

As mentioned earlier, also for PMF and ET, analysis of *JAK2* exon 14 is indispensable. In case of ET, the integrity of the *CALR* gene and the *JAK2* V617F mutation are investigated in parallel. If both results are negative, *MPL* is examined.[16] In case of PMF, NGS allows the immediate evaluation of all three driver genes, and of other genes known to be involved in myeloid neoplasms (e.g. ASXL1, EZH2, TET2, IDH1 or 2, SF3B1 and SRSF2).[2,16,22,24] Evaluation of these latter genes might become even more important in the future for prognostic purposes.[28-31]
MPL mutations are present in a small subset of ET and PMF patients (3 to 8% respectively) and typically involve exon 10. Most frequently, they constitute a replacement of tryptophan (W) at position 515 (W515L, W515K, W515R, W515A) or a serine to asparagine switch at position 505 (S505N).[32] Other variants outside exon 10 have been reported, but their clinical significance remains unclear.[12,13,21,33,34] An overview of the most frequently reported (probable) pathogenic *JAK2* and *MPL* variants is given in Appendix II.

2) What is the current status of JAK2 exon 12 and MPL exon 10 mutation analysis in UZ Leuven and how does our workflow compare to literature and other hospitals?

JAK2 exon 12

In concordance with the aforementioned guidelines, UZ Leuven performs *JAK2* exon 12 mutation analysis on EDTA bone marrow/peripheral blood samples in those patients with a strong clinical suspicion of PV who tested negative for *JAK2* V617F. The analysis is done by PCR and Sanger sequencing, enabling mutation detection from 10-15% mutated alleles onward. Only exon 12 is currently evaluated, as proposed by the guidelines.[15-18]

To estimate the performance of our current *JAK2* exon 12 screening platform, we decided to first have a look at the frequency with which *JAK2* exon 12 requests were made to the lab. Secondly, we investigated the incidence of positive *JAK2* exon 12 results. Two queries were performed, comprising all data from January 2018 to December 2018 or from the beginning of the *JAK2* exon 12 mutation analysis in 2013 until October 2019. The exact parameters of both queries can be found in Appendix IV.

In 2018, the CME received 162 samples for *JAK2* exon 12 screening from 160 patients. Only 4 of the investigated patients tested positive for exon 12 alterations (mutation rate of 2.5%).
Looking at all available data since the start of the analysis, exon 12 was found mutated in 13 out of 755 cases. The resulting low prevalence of 1.7% is in contrast to reports in literature, where most of the *JAK2* V617F negative PV patients carried exon 12 alterations.[1,7,23,35,36]
One possible explanation for this difference could be the high prevalence of *JAK2* exon 12 requests on peripheral blood samples in our institute. In UZ Leuven peripheral blood samples constitute more than half of all samples sent for exon 12 analysis (54%), while bone marrow (14%) and DNA samples (32%) constitute the other half.
Some authors have described a discrepancy between exon 12 mutant allele burden in bone marrow versus peripheral blood due to the low granulocyte involvement for these mutations.[1] However similar detection rates for both sample types have also been reported.[35,37]

Another explanation for the perceived difference, could be that papers with high incidence rates use detection methods with a higher sensitivity than our Sanger sequencing approach.
In addition, UZ Leuven receives many samples from peripheral hospitals where *JAK2* V617F analysis is sometimes still performed using PCR and Sanger sequencing. Some of the V617F positive cases could be missed if the hotspot mutation occurs at low allele frequency.[38]
In 2013, Furtado et al. reported a similarly low incidence of positive *JAK2* exon 12 cases (0.98%), which they believed to be caused by inappropriate patient selection, low mutant allele burden and rare mutations inside or outside exon 12.[39] Schnittger et al. also emphasized the importance of strict clinical characterization of PV patients, as they reported *JAK2* exon 12 alterations in 15.9% of V617F-negative PV patients, but in only 1.5% of patients with unclear erythrocytosis.[36]

Peripheral hospitals do not often perform *JAK2* exon 12 analysis. In those centers where exon 12 analysis takes place, PCR and Sanger sequencing (CHU Liège) or NGS (AZ Sint-Lucas Gent, UCL Saint-Luc) are the preferred strategies.

MPL exon 10

Patients suspected of having PMF are tested at the CME with the TruSight Myeloid panel, a commercial targeted resequencing (NGS) panel from Illumina. This panel contains primers for detection of hotspot regions in 54 genes (including *JAK2*, *CALR* and *MPL*), all involved in myeloid or -to a lesser extent- in lymphoid malignancies. Reimbursement of this test is provided by the NGS convention.
For ET, the situation is more complex. *JAK2* and *CALR* are the predominant genes involved in ET pathogenesis, so patients are tested first for hotspot regions in both genes. If these results turn out negative, *MPL* can be investigated (but only 3% of ET patients will harbor mutations in *MPL)*.
In UZ Leuven, *MPL* analysis is done by NGS since the introduction of the TruSight Myeloid panel in 2013. From 2006 until 2016, PCR and Sanger sequencing were used.
Since only the first two tests are currently reimbursed (e.g. for *JAK2* and *CALR*), *MPL* mutation analysis by NGS is quite expensive for both hospital and patient. A detailed cost analysis is provided in the next chapter.

To determine the frequency of *MPL* exon 10 requests and the mutation rate in ET and PMF cases, we performed similar queries as for the *JAK2* exon 12 test.

In 2018, 573 TruSight Myeloid panels were performed. 87 of these panels were done for patients suspected to have PMF, ET or 'MPN'. A *MPL* mutation was detected in 7 panels. The positive incidence rate was 18.8% for ET patients and 5.7% for 'MPN' patients. Only 1 patient was tested for PMF and he was wild-type for *MPL*.

Looking at all *MPL* positive TruSight results from the introduction in 2013 onwards, the prevalence of *MPL* mutations was 4.6% (24/528 requests). Surprisingly, a somewhat higher result of 8.9% (31/348) was obtained for *MPL* positive cases in the past, when PCR and Sanger sequencing were still performed. A possible explanation could be that only few patients have *MPL* exon 10 mutations at VAFs below 15 to 20%. However, additional analysis of our NGS data showed that 1 in 3 patients had VAF values <15%.
It remains unclear what causes the difference, although patient selection for *MPL* PCR in the past might have been more restrictive, while more doubtful MDS-MPN cases are now also analyzed using the TruSight kit.
Both incidence rates are well within the range described in literature (1-10%).[2, 6,12,14,40] Also the type of mutations present in our patient population corresponds well with available data, with W515L being the dominant amino acid change.[10,14,40]

NGS is most often the method of choice for *MPL* detection in other hospitals (CHU Liège, AZ Sint-Lucas Gent, UCL Saint-Luc), while AZ Sint-Jan Brugge still uses PCR and Sanger sequencing for variant detection.

Relevant data from the *JAK2* and *MPL* prevalence study are summarized in Appendix V.

3) Is allele-specific PCR a valuable option for JAK2 exon 12 and/or MPL exon 10 mutation analysis?

Due to the high sensitivity threshold of our current *JAK2* exon 12 protocol and the high cost related to *MPL* exon 10 determination, we decided to evaluate whether AS-PCR could be a suitable alternative approach. In AS-PCR one or both primers are designed to specifically target the altered sequence region, so that primer annealing and amplification will only occur in case the targeted genetic modification is present.
AS-PCR is relatively cheap, can screen multiple genetic variants in parallel and has a very high analytical sensitivity. Disadvantages however are that the number of detectable variants is limited (usually only hotspot regions are investigated) and that polymorphisms affecting primer aligning regions may cause false negative results.

In 2013, Furtado et al. published two papers describing AS-PCR methods for *JAK2* exon 12 and *MPL* exon 10 analysis.[39,41] We were able to adapt both protocols so that PCR reagents and settings were suitable for both *JAK2* and *MPL* testing, and so that the AS-PCR could run in parallel with our standard *CALR* PCR. Fragment analysis was performed on an ABI3730xl, followed by interpretation of the results using GeneMapper.

The following primers and PCR conditions were eventually applied:

Primer	Sequence
JAK2-E12-forward	5'-/56-FAM/CTCCTCTTTGGAGCAATTCA-3'
JAK2-E12-reverse	5'-TCCAATGTCACATGAATGTAAATC-3'
JAK2-E12-K539L-forward	5'-/5HEX/GAACCAAATGGTGTTTCACTT-3'
MPL-forward	5'-/56-FAM/TGGGCCGAAGTCTGACCCTTT-3'
MPL-reverse	5'-CAGAGCGAACCAAGAATGCCTGT-3'
MPL-W515L-forward	5'-/56-FAM/GGCCTGCTGCTGCTGAGATT-3'
MPL-W515K-forward	5'-/56-FAM/GCCTGCTGCTGCTGAGGAA-3'
MPL-W515A-reverse	5'-GTAGTGTGCAGGAAACTGCGC-3'
MPL-S505N-reverse	5'-CAGGCCCAGGACGGCGT-3'

Primers were designed to detect common indels such as N542_E543del, E543_D544del, F537_K539delinsL or others, and point mutation K539L in *JAK2* exon 12, and for detection of W515L, W515K, W515A or S505N mutations in *MPL* exon 10.

PCR program:

Temperature	Duration	Cycles
95°C	15 min	1
95°C	40 sec	35
60°C	60 sec	
72°C	60 sec	
72°C	7 min	1
4°C	∞	1

We selected 30 patient samples to test our AS-PCR protocol, 10 for *JAK2* exon 12 and 20 for *MPL* exon 10 analysis. Four out of ten samples selected for *JAK2* analysis had wild type exon 12 sequence. One patient carried the K539L point mutation, while the remaining 5 patients harbored a deletion (2), insertion (1) or combination of both (2). Overall correlation was 10 of 10 (100%). One patient (case 6) had a N542_E543del that was not detected at first with standard Sanger sequencing but was picked up with NGS after a specific request was made by the treating physician. AS-PCR however identified the deletion faultlessly.
It is highly likely that, with additional testing, AS-PCR will uncover even more false negative results from Sanger sequencing. Preliminary dilution experiments of samples with a known mutant allele burden point at a detection threshold of 2% or even lower for AS-PCR. This is well below the sensitivity threshold of 10-15% for Sanger sequencing.
A downside of AS-PCR compared to Sanger sequencing is that copy number variations are not specified to the nucleotide level but are reported in a more general way (as 'deletion' or 'insertion'). The exact position of your fragment peak however gives some clue about the type of indel present, especially for the most typical ones.

Table Selected patients for *JAK2* exon 12 and *MPL* exon 10 AS-PCR testing

Case	Gene	Mutation	VAF (dilution minimum)	Result AS-PCR	Discordant
1	*JAK2* exon 12	Wild type		Wild type	
2	*JAK2* exon 12	Wild type		Wild type	
3	*JAK2* exon 12	Wild type		Wild type	
4	*JAK2* exon 12	Wild type		Wild type	
5	*JAK2* exon 12	K539L	/	K539L	
6	*JAK2* exon 12	N542_E543del	12% (2%)	Deletion	
7	*JAK2* exon 12	N542_E543del	50%	Deletion	
8	*JAK2* exon 12	H538_K539delinsL	/	Deletion	
9	*JAK2* exon 12	N542_E543delinsK	/	Deletion	
10	*JAK2* exon 12	I546_F547ins11	/	Insertion	
11	*MPL* exon 10	Wild type		Wild type	
12	*MPL* exon 10	Wild type		Wild type	
13	*MPL* exon 10	Wild type		Wild type	
14	*MPL* exon 10	Wild type		Wild type	
15	*MPL* exon 10	W515R	35%	Wild type	Not included in assay
16	*MPL* exon 10	W515L	2%	W515L	
17	*MPL* exon 10	W515L	48%	W515L	
18	*MPL* exon 10	W515L	34%	W515L	
19	*MPL* exon 10	W515L	7%	W515L	
20	*MPL* exon 10	W515L	3%	W515L	
21	*MPL* exon 10	W515L	2%	W515L	Not called, but small peak visible
22	*MPL* exon 10	S505N	23% (2%)	S505N	
23	*MPL* exon 10	S505N	45%	S505N	
24	*MPL* exon 10	W515K	24% (2%)	W515K	
25	*MPL* exon 10	W515K	43%	Wild type	Indel leading to W515K instead of point mutation
26	*MPL* exon 10	W515K	/	W515K	
27	*MPL* exon 10	W515A	43% (2%)	W515A	
28	*MPL* exon 10	W515A	17%	W515A	
29	*MPL* exon 10	W515L & R514K	/	Wild type	No primer annealing due to R514K
30	*MPL* exon 10	Intron 10	/	Wild type	Not included in assay, no known clinical significance

For *MPL* analysis, 20 samples were collected containing some wild type sequence (4), point mutation W515L (6), W515K (3), W515A (2) and S505N (2). We also selected 3 patients with rare *MPL* variants. One patient carried a W515R change, not detectable with the primers included in the AS-PCR protocol. As expected, this mutation was not picked up and should be considered a true false negative. Another false negative result was obtained for patient 29, who suffered two adjacent point mutations (R514K and W515L) disrupting primer annealing and amplification of the W515L amplicon. A third false negative result was caused by an indel leading to the W515K mutation instead of the typical point mutation (patient 25).
In a fourth patient, Sanger sequencing was still performed (the sample dated from the very start of the TruSight myeloid panel) that revealed a single nucleotide change in intron 10. This variant was not detectable with AS-PCR, but neither would it have been with NGS. There is also no known clinical significance linked to the alteration.
The number of false negative results therefore remains 3 out of 19 samples or 15.8%. In real life, the rate of false negatives will be much lower since we specifically selected these rare *MPL* variants for our experiments.
We encountered no false positive results for the *MPL* exon 10 analysis, nor for the *JAK2* exon 12 AS-PCR.
The detection threshold of mutated *MPL* was 2% or lower, but further evaluation is needed. One patient (case 21) had a VAF of 2% for a W515L mutation that was not called by the GeneMapper algorithm. Visual inspection however could clearly distinguish a small peak at the right location. Dilution series will help to establish the true limit of detection for the AS-PCR protocol.

If AS-PCR instead of NGS had been used from the start to detect *MPL* exon 10 alterations, 4 out of 55 *MPL*-positive patients (7% or fewer than 1 each year) would have been misdiagnosed as wild type. All of them having rare, atypical *MPL* mutations. No *JAK2* exon 12 alterations (0 out of 14) would have been missed.

Moreover, due to the lower detection limit of AS-PCR compared to standard PCR, it is likely that more *JAK2* positive cases would have been detected, as was shown for patient 6.

Probably the most convincing argument still for implementation of the AS-PCR in routine practice is the reduction in expenses for the hospital. AS-PCR is a bit more expensive than standard PCR due to the development of fluorophore labeled primers (60 euro compared to only a few euro). This cost however only needs to be done once or a few times each year since very few amount of stock primers is needed for a single test. The true saving of course is in the reduction of the number of NGS analyses. Reimbursement for NGS is 350 euro, but the true cost probably lies in between 350 and 500 euro. Furthermore, NGS for ET is not reimbursed at all, although this might change in the near future.

For 2018 alone, in between 30 100 to 43 000 euro was spent to perform 86 TruSight panels for patients with suspected ET or 'MPN'. If we estimate the cost of AS-PCR at 90 euro per test, an expense of 7740 euro would have been made instead. This means a saving of at least 22 360 euro.
Besides the enormous cost reduction, there is also a substantial reduction in time and personnel requirements. AS-PCR for *MPL* and *JAK2* together with *CALR* PCR can be run by a single technician on the same day, inside the same PCR machine, with results available only one day later. Interpretation of the results is also more straightforward than for NGS.

Table Overview cost estimation of NGS versus AS-PCR

This table is only available after a specific request is made to the author.

In summary, AS-PCR is a valuable option for *JAK2* exon 12 and *MPL* exon 10 mutational analysis, as it leads to a significant reduction in both workload and lab expenses. There are however inherent limitations to this approach. AS-PCR will give false negative results when variants are present that affect primer annealing regions or when mutations are located outside the region of interest and therefore not evaluated.
In the future, it might again prove worthwhile to perform NGS for all cases of suspected MPN when the role and involvement of other variants and genes becomes elucidated. And of course, if reimbursement is instated.

COMMENTS

I would like to thank my colleague Annelies Louwagie for updating the AS-PCR protocol so that it matched the criteria for *CALR* PCR and all three gene analyses (*JAK2*, *MPL*, *CALR*) can be completed in a single run.

TO DO/ACTIONS

1) Dilution experiments for both *JAK2* exon 12 and *MPL* exon 10 to determine the limit of detection
2) Start validation of the assay for implementation in routine practice
3) Inform clinicians (UZ Leuven and peripheral hospitals) about the new workflow

ATTACHMENTS

Appendix I: WHO criteria for PV, ET and PMF (adapted from *Arber DA,* et al. Blood 2016)

WHO criteria for PV

WHO PV criteria
Major criteria
1. Hemoglobin >16.5 g/dL in men
Hemoglobin >16.0 g/dL in women
or,
Hematocrit >49% in men
Hematocrit >48% in women
or,
increased red cell mass (RCM)*
2. BM biopsy showing hypercellularity for age with trilineage growth (panmyelosis) including prominent erythroid, granulocytic, and megakaryocytic proliferation with pleomorphic, mature megakaryocytes (differences in size)
3. Presence of *JAK2*V617F or *JAK2* exon 12 mutation
Minor criterion
Subnormal serum erythropoietin level
Diagnosis of PV requires meeting either all 3 major criteria, or the first 2 major criteria and the minor criterion†

* More than 25% above mean normal predicted value.

† Criterion number 2 (BM biopsy) may not be required in cases with sustained absolute erythrocytosis: hemoglobin levels >18.5 g/dL in men (hematocrit, 55.5%) or >16.5 g/dL in women (hematocrit, 49.5%) if major criterion 3 and the minor criterion are present. However, initial myelofibrosis (present in up to 20% of patients) can only be detected by performing a BM biopsy; this finding may predict a more rapid progression to overt myelofibrosis (post-PV MF).

WHO criteria for ET

WHO ET criteria
Major criteria
1. Platelet count ≥450 × 10^9/L
2. BM biopsy showing proliferation mainly of the megakaryocyte lineage with increased numbers of enlarged, mature megakaryocytes with hyperlobulated nuclei. No significant increase or left shift in neutrophil granulopoiesis or erythropoiesis and very rarely minor (grade 1) increase in reticulin fibers
3. Not meeting WHO criteria for *BCR-ABL1*$^+$ CML, PV, PMF, myelodysplastic syndromes, or other myeloid neoplasms
4. Presence of *JAK2*, *CALR*, or *MPL* mutation
Minor criterion
Presence of a clonal marker or absence of evidence for reactive thrombocytosis
Diagnosis of ET requires meeting all 4 major criteria or the first 3 major criteria and the minor criterion

WHO criteria for prePMF

WHO prePMF criteria
Major criteria
1. Megakaryocytic proliferation and atypia, without reticulin fibrosis >grade 1*, accompanied by increased age-adjusted BM cellularity, granulocytic proliferation, and often decreased erythropoiesis
2. Not meeting the WHO criteria for *BCR-ABL1*+ CML, PV, ET, myelodysplastic syndromes, or other myeloid neoplasms
3. Presence of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker,† or absence of minor reactive BM reticulin fibrosis‡
Minor oriteria
Presence of at least 1 of the following, confirmed in 2 consecutive determinations:
a. Anemia not attributed to a comorbid condition
b. Leukocytosis ≥11 × 10^9/L
c. Palpable splenomegaly
d. LDH increased to above upper normal limit of institutional reference range
Diagnosis of prePMF requires meeting all 3 major criteria, and at least 1 minor criterion

† In the absence of any of the 3 major clonal mutations, the search for the most frequent accompanying mutations (eg, *ASXL1*, *EZH2*, *TET2*, *IDH1/IDH2*, *SRSF2*, *SF3B1*) are of help in determining the clonal nature of the disease.

‡ Minor (grade 1) reticulin fibrosis secondary to infection, autoimmune disorder or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies.

WHO criteria for overt PMF

WHO overt PMF oriteria
Major oriteria
1. Presence of megakaryocytic proliferation and atypia, accompanied by either reticulin and/or collagen fibrosis grades 2 or 3*
2. Not meeting WHO criteria for ET, PV, *BCR-ABL1*+ CML, myelodysplastic syndromes, or other myeloid neoplasms
3. Presence of *JAK2*, *CALR*, or *MPL* mutation or in the absence of these mutations, presence of another clonal marker,† or absence of reactive myelofibrosis‡
Minor oriteria
Presence of at least 1 of the following, confirmed in 2 consecutive determinations:
a. Anemia not attributed to a comorbid condition
b. Leukocytosis ≥11 × 10^9/L
c. Palpable splenomegaly
d. LDH increased to above upper normal limit of institutional reference range
e. Leukoerythroblastosis
Diagnosis of overt PMF requires meeting all 3 major criteria, and at least 1 minor criterion

† In the absence of any of the 3 major clonal mutations, the search for the most frequent accompanying mutations (eg, *ASXL1*, *EZH2*, *TET2*, *IDH1/IDH2*, *SRSF2*, *SF3B1*) are of help in determining the clonal nature of the disease.

‡ BM fibrosis secondary to infection, autoimmune disorder, or other chronic inflammatory conditions, hairy cell leukemia or other lymphoid neoplasm, metastatic malignancy, or toxic (chronic) myelopathies.

Appendix II: Overview of (probable) pathogenic *JAK2* and *MPL* variants

JAK2

Mutation	Exon	Frequency	Nucleotide position	Reference
L624P	15	≈1/20000 MPN	c.1871T>C	27
I645V	15	≈1/20000 MPN	c.1933A>G	27
V617F	14	~96% PV, ~55% ET, ~65% PMF	c.1849G>T	17
V617F,C618R	14	<1% PV	c.1849G>T, c.1851C>T, c.1852T>C	17
V617F,C618F	14	<1% ET	c.1849G>T, c.1853G>T	17
V617F,D620E	14	<1% PV	c.1849G>T, c.1860C>A	17
L611V,V617F	14	<1% PV	c.1831T>G, c.1849G>T	17
C616C,V617F	14	<1% ET	c.1848T>C, c.1849G>T	17
D620E	14	<1% MPN	c.1860C>A	17
L583_A586delinsS	13	≈2/174 PV/CEL	c.1747_1756delinsT	26
F557Fs	13	≈1/20000 MPN	c.1671_1672del	27
R564L	13	≈3/20000 MPN	c.1691G>T	27
R564Q	13	≈2/20000 MPN	c.1691G>A	27
V567A	13	≈1/20000 MPN	c.1700T>C	27
G571S	13	≈3/20000 MPN	c.1711G>A	27
G571R	13	≈1/20000 MPN	c.1711G>C	27
L579F	13	≈1/20000 MPN	c.1735C>T	27
H587N	13	≈1/20000 MPN	c.1759C>A	27
S591L	13	≈1/20000 MPN	c.1772C>T	27
V536_I546dup	12	<1% V617F-negative PV	c.1606_1638dup33	17
V536_F547dup	12	<1% V617F-negative PV	NA	17
F537I,K539I	12	<1% V617F-negative PV	c.1609T>A, c.1616A>T	17
F537_K539delinsL	12	~10% V617F-negative PV	c.1609_1616delins	17
F537_I546dup10,F547L	12	<1% V617F-negative PV	c.1608_1640dup33	17
F537_547dup	12	<1% V617F-negative PV	c.1609_1641dup33	17
H538Q,K539L	12	<1% V617F-negative PV	c.1614C>A, c.1615A>T, c.1616A>T	17
H538D,K539L,I540S	12	<1% V617F-negative PV	NA	17
H538_K539del	12	<1% V617F-negative PV	NA	17
H538_K539delinsF	12	<1% V617F-negative PV	c.1612_1617delins	17
H538_K539delinsI	12	<1% V617F-negative PV	c.1612_1616delins	17
H538_K539delinsL	12	~5% V617F-negative PV	c.1612_1617delins	17
K539L	12	~10% V617F-negative PV	c.1615A>T or A>C, c.1616A>T	17
K539L,L545V	12	<1% V617F-negative PV	c.1615A>T, c.1633T>G	17
I540T	12	<1% V617F-negative PV	c.1619T>C	17
I540_N542delinsS	12	<1% V617F-negative PV	NA	17
I540_E543delinsMK	12	~5% V617F-negative PV	c.1620_1627delins	17
I540_E543delinsKK	12	<1% V617F-negative PV	c.1619_1627delins	17
R541_E543delinsK	12	~10% V617F-negative PV	c.1622_1627delins	17
R541K,A542_G543del	12	<1% V617F-negative PV	c.1622G>A, c.1624_1629del	17
R541_E543delinsK	12	<1% V617F-negative PV	c.1622_1627delins	17
N542_E543del	12	~40% V617F-negative PV	c.1624_1629del	17
E543_D544del	12	~10% V617F-negative PV	c.1627_1632del	17
D544G	12	<1% V617F-negative PV	c.1631A>G	17
D544_L545del	12	<1% V617F-negative PV	c.1630_1635del	17
F547_K549delinsL	12	<1% V617F-negative PV	NA	17
547insL,I540_F547dup8	12	<1% V617F-negative PV	c.1642_1644ins, c.1645_1668dup	17

Mutation	Exon	Frequency	Nucleotide position
W515L or W515K	10	~5% PMF	c.1544G>T
		~3% ET	c.1543T>A
			c.1544G>A
V501A,W515L	9, 10	<1% PMF	c.1502T>C
			c.1544G>T
W501A,W515R	9, 10	<1% PMF, ET	c.1502T>C
			c.1543T>C
S505C,W515L	10	<1% ET	c.1514G>A
			c.1544G>T
S505N	10	<1% PMF, ET	c.1514G>A
A506T	10	<1% PMF	c.1516G>A
L510P	10	<1% PMF	c.1529T>C
W515A	10	<1% ET	c.1543T>G
			c.1544G>C
A519T	10	<1% PMF	c.1555G>A
S204P	4	<1% PMF	c.610T>C
Y252H	5	<1% ET	c.754T>C

Appendix III: Sensitivity of different technologies used for clinical molecular diagnostics (adapted from *Gong JZ,* et al. J Mol Diagn 2013 and *Palumbo GA,* et al. Front Oncol 2019)

Method	Benefits	Critical points	Sensitivity (%)
qPCR (AS, LNA)	High sensitivity; quantitative	Detects only target mutations	0.1−0.01
PCR (AS)	High sensitivity; simple to perform	Detects only target mutations; not quantitative	0.1−1
Melting curve analysis	Simple to perform; semiquantitative; low cost	Detects target mutation only; moderate to low sensitivity; poor reproducibility in low+ samples	5−10
Pyrosequencing	Simple to perform; quantitative; low cost	Detects target mutation only; relatively low sensitivity	5−10
RFLP	Low cost	Relatively low sensitivity; requires post-PCR manipulation; unreliable in low+ samples; not quantitative.	1−10
Sanger sequencing	Detects known and unknown mutations; bidirectional confirmation; validated methods	Low sensitivity; time-consuming; not quantitative; high input of DNA/RNA	10-20
Real time PCR	Detection of known mutations; validated methods	High input of DNA/RNA; no simultaneous screening of multiple genes in multiple samples	1
Digital PCR	Low input of DNA/RNA; detection of known mutations; cost-effective for rapid genotyping and monitoring	No simultaneous screening of multiple genes in multiple samples	0.1-1
NGS	Low input of DNA/RNA; massively parallel sequencing; decreased sequencing cost/gene; detection of known and unknown mutations; simultaneous screening of multiple genes in multiple samples	Validation studies required; high-complexity workflow and analyzing results; genome data analysis is time-consuming	1

PCR, polymerase chain reaction; AS, allele specific; LNA, locked nucleic acid; RFLP, restriction fragment length polymorphism; SS, Sanger sequencing; NGS, next generation sequencing

Appendix IV: Query parameters

Query *JAK2* exon 12

"resultaten Afw ZV PriKol" (760) {van: 01-01-2018, tot: 31-12-2018, bronnen: [BRON:LIS/Laboratoria UZ Leuven], vereist: [ENTITEIT:MD2: JAK2 exon 12 (beenmerg/bloed/diverse/klier/tumor): resultaat (P)], voorlopig: false, annulaties: false, pseudos: false, eenheden: true, afwerking: false, samenvoegen: niet, server: MANAGEMENT}

"resultaten Afn ZV PriKol" (610) {van: 01-01-2000, tot: 02-10-2019, bronnen: [BRON:LIS/Laboratoria UZ Leuven], vereist: [ENTITEIT:MD2: JAK2 exon 12 (beenmerg/bloed/diverse/klier/tumor): resultaat (P)], voorlopig: false, annulaties: false, alle statussen: false, pseudos: false, eenheden: true, samenvoegen: niet, server: MANAGEMENT}

"resultaten Afn ZV PriKol" (610) {van: 01-01-2000, tot: 02-10-2019, bronnen: [BRON:CME Archief (UZ Leuven)], vereist: [ENTITEIT:MD2: JAK2 exon 12 (diverse): resultaat (P)], voorlopig: false, annulaties: false, alle statussen: false, pseudos: false, eenheden: true, samenvoegen: niet, server: MANAGEMENT}

"resultaten Afn ZV PriKol" (610) {van: 01-01-2000, tot: 02-10-2019, bronnen: [BRON:LIS/Laboratoria UZ Leuven], vereist: [ENTITEIT:MYELOID (beenmerg/bloed/diverse/klier/tumor): gen, ENTITEIT:MYELOID (beenmerg/bloed/diverse/klier/tumor): mutatie], voorlopig: false, annulaties: false, alle statussen: false, pseudos: false, eenheden: true, samenvoegen: niet, server: MANAGEMENT}

Query *MPL* exon 10

"resultaten Afn ZV PriKol" (610) {van: 01-01-2018, tot: 31-12-2018, bronnen: [BRON:LIS/Laboratoria UZ Leuven], vereist: [ENTITEIT:MYELOID (beenmerg/bloed/diverse/klier/tumor): panel, ENTITEIT:MYELOID (beenmerg/bloed/diverse/klier/tumor): genotype], voorlopig: false, annulaties: false, alle statussen: false, pseudos: false, eenheden: true, samenvoegen: niet, server: MANAGEMENT}

"resultaten Afn ZV PriKol" (610) {van: 01-01-2000, tot: 02-10-2019, bronnen: [BRON:CME Archief (UZ Leuven)], vereist: [ENTITEIT:MD2: MPL exon 10 (diverse): resultaat (P)], voorlopig: false, annulaties: false, alle statussen: false, pseudos: false, eenheden: true, samenvoegen: niet, server: MANAGEMENT}

"resultaten Afn ZV PriKol" (610) {van: 01-01-2000, tot: 02-10-2019, bronnen: [BRON:LIS/Laboratoria UZ Leuven], vereist: [ENTITEIT:MYELOID (beenmerg/bloed/diverse/klier/tumor): gen, ENTITEIT:MYELOID (beenmerg/bloed/diverse/klier/tumor): mutatie], voorlopig: false, annulaties: false, alle statussen: false, pseudos: false, eenheden: true, samenvoegen: niet, server: MANAGEMENT}

Appendix V: Results from the query regarding *JAK2* exon 12 and *MPL* exon 10 testing

JAK2 exon 12

January 2018 – December 2018: *JAK2* exon 12 requests

Method	Number of patients tested	*JAK2* positive (%)
PCR + Sanger sequencing	160	4 (2.5%)

January 2013 - October 2019: *JAK2* exon 12 requests

Method	Number of patients tested	*JAK2* positive (%)
PCR + Sanger sequencing	755	13 (1.7%)
NGS	1	1

January 2013 - October 2019: type of samples received

Sample type	Number of samples (% of total)	*JAK2* positive (% of total)
Bone marrow or DNA derived from bone marrow	110 (14.4)	3 (21.4)
Peripheral blood or DNA derived from peripheral blood	409 (53.5)	7 (50.0)
DNA not otherwise specified	245 (32.0)	4 (28.6)
other	1 (0.1)	0 (0.0)
total	765	14

January 2013 - October 2019: type of *JAK2* mutations detected

Variant	Reported in LWS (% of total)
N542_E543del	5 (35.7)
E543_D544del	0 (0.0)
K539L	3 (21.4)
F537_K539delinsL	1 (7.1)
R541_E543delins	2 (14.3)
H538_K539delinsL	1 (7.1)
I546_F547ins11	1 (7.1)
F537I + K539I	1 (7.1)
Total	14

January 2018 – December 2018: TruSight myeloid panel requests

Indication	Number of panels (% of total)	*MPL* positive (%)
ET	16 (2.8)	2 (12.5)
PMF	1 (0.2)	0 (0.0)
MPN	70 (12.2)	4 (5.7)
Other	486 (84.8)	1 (0.2)
total	573	7

January 2013 - October 2019: TruSight myeloid panel requests

Indication	Number of panels (% of total)	*MPL* positive (%)
ET	24 (0.8)	2 (8.3)
PMF	45 (1.5)	2 (4.4)
MPN	420 (14.2)	20 (4.8)
MDS/MPN-RS	31 (1.0)	0 (0.0)
MDS/MPN	11 (0.4)	0 (0.0)
other	2426 (82.0)	5 (0.2)
total	2957	29

January 2013 - October 2019: standard PCR and Sanger sequencing for *MPL* exon 10

	Number	*MPL* positive (%)
Samples	362	33 (9.1)
Patients	348	31 (8.9)

January 2013 - October 2019: type of *MPL* mutations detected

Variant	Reported in LWS (% of total)	Research setting (% of total)
W515L	32 (69.6)	10 (62.5)
W515K	3 (6.5)	4 (25.0)
W515A	2 (4.3)	0 (0.0)
W515R	3 (6.5)	0 (0.0)
S505N	2 (4.3)	0 (0.0)
Intron 10	4 (8.7)	1 (6.3)
W515L + R514K	0 (0.0)	1 (6.3)
Total	46	16