

**Pilot Myeloid Gene Panels – FINAL REPORT
(Not Accredited)**

Distribution – 232401

Date Issued – 30 Oct 2023

Participant –

Closing Date – 22 Dec 2023

IMPORTANT: Please note this individual participant section (v1.0.1) of the final trial report replaces the interim report v1.0.0 (dated 27 March 2024). There is no change in content for this section of the report (pages 1-11). However, a detailed breakdown of nomenclature and educational aspects relating to variant interpretation is now available in the addendum section (v1.0.0) of this finalised trial report (pages i-vii).

Trial Comments

This trial was issued to 131 participants; 124 (94.7%) laboratories returned results. Of the 7 participants failing to submit results, two laboratories pre-notified us of this.

Sample Comments

The genomic DNA sample (Myeloid GP 116) was extracted from the peripheral blood of an adult patient with a working diagnosis of myeloproliferative neoplasm (MPN), essential thrombocythaemia (ET), and distributed by UK NEQAS LI. The material was potentially processed >48 hours following collection.

Your Laboratory Record status for this trial:	
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IMPORTANT: To permit meaningful trial data analysis it is essential the information held in your Laboratory Record is complete and accurately reflects your current practice in relation to this programme. Please provide all the information as requested and/or check it carefully to ensure methodological details are up to date when requested to do so. We will be next asking for all Laboratory Record information to be re-submitted for trial Myeloid GP 242501.

Sample Myeloid GP 116

Your Results – Variant(s) of strong clinical significance

Gene	Your DNA sequence variant detected	Your protein variant

Your Results – Variant(s) of potential clinical significance

Gene	Your DNA sequence variant detected	Your protein variant

Your Results – Variant(s) of unknown clinical significance

Gene	Your DNA sequence variant detected	Your protein variant

Classification terminology based on Li *et al.* (2017) Joint consensus recommendations from the Association for Molecular Pathology, American Society of Clinical Oncology and College of American Pathologists¹.

Please note, due to formatting limitations some rows may appear blank within the tables(s) above. All submitted variant(s) of unknown clinical significance may not be reflected in the above table for individual participants due to formatting and space constraints.

All Participant Results

In the interests of clarity, we have only summarised variants reported by ≥5 participants in the table below.

Gene	n ^a	Variant classification ^b			Variant detected (consensus) ^c		Median VAF % (IQR) ^d
		Strong clinical significance	Potential clinical significance	Unknown clinical significance	DNA sequence description	Protein level description	
MPL	61/115	22	21	18	NM_005373.3: c.610T>C	p.(Ser204Pro)	11.8 (1.2)
CBL	8/102	4	3	1	NM_005188.4: c.1211G>A	p.(Cys404Tyr)	1.0 (0.2)

^a Total number of participants reporting this variant/number of participants stating the inclusion of the relevant gene on their panel or known to feature the gene on their panel due to identification of the consensus variant. Please note for this trial 10 returning participants failed to provide full Laboratory Record information. Not all laboratories provided sufficient gene/region of interest information for their panel to permit identification of all false negative results in the data set. Additionally, please refer to the report comments section (once published) regarding any participant(s) reporting a consensus variant from a gene not stated as included on their panel.

^b Based on Li *et al.* (2017) Joint consensus recommendations from the Association for Molecular Pathology, American Society of Clinical Oncology and College of American Pathologists¹.

^c Nomenclature provided in the table is based on the MANE Select (v1.0)² reference transcript and genome build GRCh38, unless specified. Please refer to the comments section (once published) for further information about reference sequences. Results returned by participants, at both the DNA and protein level, may have been harmonised to the equivalent Human Genome Variation Society (HGVS) approved nomenclature (version 20.05)³.

^d during the compilation of the 'All Participant Results' table. Information regarding a variant(s) reported in any gene listed in the table, which could not be identified as equivalent to a consensus variant has been excluded. Protein nomenclature includes parentheses as it represents a prediction from analysis at the DNA level.

^d Descriptive statistics calculated for any variant with >2 quantification data points. Median VAF calculated for DNA based assays, all panels and platforms. Percentage values quoted have been subjected to rounding up/down to 1 d.p., IQR = interquartile range. Quantitative data points may have been excluded from the statistics if the associated nomenclature provided was considered equivocal.

Your Performance

Performance	Performance Status for this Sample	Performance Status Classification Over 3 Sample Period	
		Satisfactory	Critical
n/a	n/a	n/a	n/a

Please note: This programme is not currently performance monitored. We will work towards a scoring system as the programme develops.

Methods

Please note figures in the tables below may not tally with the total number of participants returning results due to some participants not returning all data requested or using multiple techniques. At the time of reporting, 10 returning participants failed to provide all the Laboratory Record information requested.

Methodological approach

	Returns
Targeted Gene Panel (DNA seq)	99
Targeted Gene Panel (DNA with RNA fusion transcript seq)	15

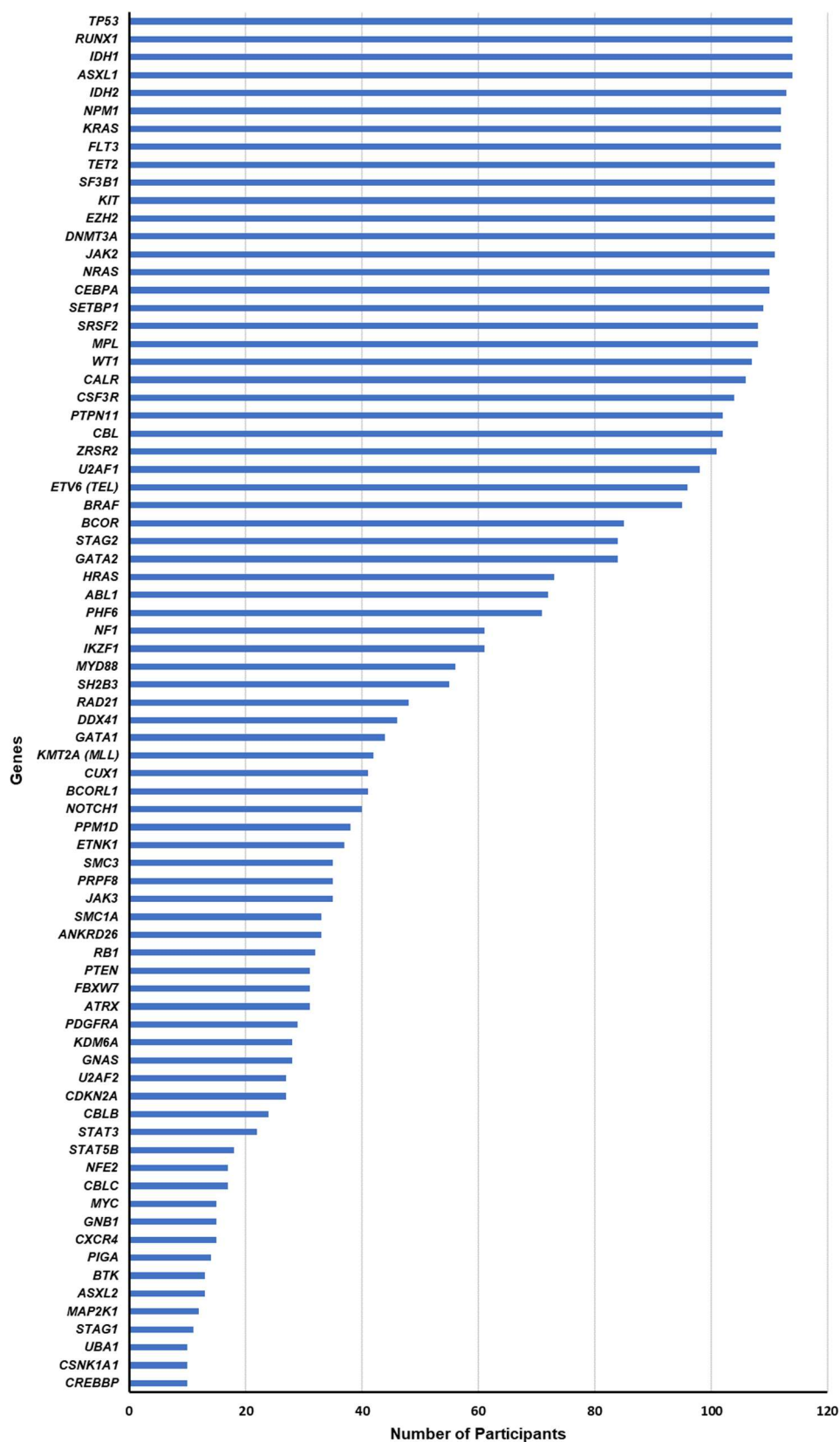
NGS platform(s) used (to analyse the sample in this trial)

	Returns
Illumina MiSeq	41
Illumina NextSeq	31
Thermo Fisher Scientific Ion Torrent (Life Tech) Ion S5	14
Illumina Novaseq	12
Illumina MiniSeq	10
Thermo Fisher Scientific Ion Torrent Genexus system	5
Thermo Fisher Scientific Ion Torrent (Life Tech) Ion S5 XL	2
Illumina iSeq 100	2
Element Bioscience AVITI	1

NGS panel description (to analyse the sample in this trial)

	Returns
(IonTorrent) Oncomine Myeloid Research Assay	18
Sophia Genetics Myeloid Solution (MYS)	17
In house (capture based)	13
Other	11
Qiagen QIASeq Custom Panel	10
Illumina TruSight Myeloid Sequencing Panel	10
Archer VariantPlex Myeloid panel	6
Twist Custom Panel	6
In house (amplicon based)	5
Sophia Genetics Extended Myeloid Solution	4
AmpliSeq for Illumina Myeloid Panel	4
Agilent SureSelect Custom QXT Panel	4
Oncomine™ Myeloid DNA Assay GX v2- all genes included in the kit	3
Illumina TruSight Oncology 500 (TSO500) Panel	2
Imegen Haematology OncoKitDx	2

Genes routinely analysed by participants (in this clinical context). Information provided by 114 laboratories; data is presented as submitted by participants (and not subject to comprehensive cross checking with reference to variant(s) detected results from individual laboratories). Only genes routinely analysed by at least 10 participants are represented in the chart.



Genome Assembly

	Returns
GRCh37/hg19	98
GRCh38	16

Minimum variant allele frequency (VAF) for reporting identification of an indel (deletion/duplication/insertion) variant

	Returns
<1%	1
1%	12
>1-2%	14
>2-3%	12
>3-4%	4
>4-5%	70
10%	1

Minimum variant allele frequency (VAF) for reporting identification of a single nucleotide variant (SNV) or substitution variant

	Returns
1%	18
>1-2%	14
>2-3%	14
>3-4%	4
>4-5%	64

Annotation database resources

	Returns
COSMIC (Catalogue Of Somatic Mutations In Cancer)	111
ClinVar (NCBI)	105
The TP53 Database (National Cancer Institute) [previously WHO IARC TP53 Database]	65
OncoKB (Memorial Sloan Kettering Cancer Center <i>et al.</i>)	43
OMIM (NCBI)	36
My Cancer Genome (Vanderbilt-Ingram Cancer Center)	32
Seshat (TP53) Database	32
CIVIC (Clinical Interpretation of Variants in Cancer)	25
The Clinical Knowledgebase (CKB) Jackson Laboratory (Boost)	23
HGMD (The Human Gene Mutation Database)	23
cBioPortal (Memorial Sloan Kettering Cancer Center <i>et al.</i>)	22
The Cancer Genome Atlas (TCGA)	20
UMD (TP53) Database	16

As stated by ≥3 participants.

Large population dataset/resources routinely consulted

	Returns
gnomAD (Genome Aggregation Database)	99
dbSNP (Short Genetic Variations, NCBI)	84
1000 Genomes	53
ESP (Exome Sequencing Project, NHLBI GO)	32

As stated by ≥3 participants.

Aggregation tool(s) utilised to access annotation resources

	Returns
Varsome (SAPHETOR)	46
Alamut (SOPHiA GENETICS)	42
Franklin (GENOOX)	22

As stated by ≥3 participants.

Trial Comments

Methodology

- All returning participants (with the relevant information provided in their Laboratory Record) described the application of a DNA based targeted gene panel NGS testing approach (n=114). At least 15 laboratories stated the additional inclusion of RNA fusion gene transcript sequencing. Please note, for this programme laboratories are not requested to report large changes affecting genome architecture or copy number variants (>50 kb).
- The average number of genes currently analysed by laboratories on a given panel is 45 with a range of 8 – 186 genes in total. For the genes most frequently included on participant gene panels (and analysed in this clinical context) please refer to the chart on page 6.
- Overall, 81.4% (n=96) of returning participants (providing the relevant information) employed bridge amplified reversible dye terminator-based platforms from Illumina to analyse sample Myeloid GP 116. The remaining laboratories stated the use of Thermofisher Scientific Ion Torrent (n=21) or Element Bioscience technology (n=1).
- The most utilised 'off the shelf' commercially available panel kits included the Ion Torrent OncoPrint Myeloid Research Panel (n=18), Sophia Genetics Myeloid Solution (n=17), Illumina TruSight Myeloid Sequencing Panel (n=10) and Archer VariantPlex Myeloid Panel (n=6).
- The proportion of returning participants (providing the relevant information) working to the GRCh38 human genome assembly was 14.0% (n=16), all Illumina platforms users.

Annotation and interpretation

- COSMIC (n=111), ClinVar (n=105) and gnomAD (n=99) remain the annotation resources most widely employed by participants. Please refer to the tables on page 8 for further information.
- Growth in the use of Franklin (GENOOX) (n=22) continues. Along with other established aggregation tools including Varsome (SAPHETOR) (n=46) and Alamut (SOPHiA GENETICS) (n=42), such approaches can be extremely useful but should always be employed with caution. Submissions to resource databases may not be subject to a level of curation sufficient for clinical diagnostic application; it is prudent to check the underpinning publication and/or supporting source information. Many resources access the same primary dataset(s); laboratories are encouraged to be mindful of duplicated evidence when classifying variants in terms of biological and/or clinical significance.
- Regarding assay performance, 60.5% (n=69) and 55.3% (n=63) of responding laboratories stated application of a 5% minimum variant allele frequency (VAF) threshold for reporting identification of a deletion/duplication/insertion (indel) and single nucleotide variant (SNV) (substitution), respectively. For an indel, 38.6% (n=44) of participants quoted a minimum threshold below 5%. For an SNV the thresholds were set lower with 44.7% (n=51) laboratories applying a minimum VAF below 5%.

Sample Myeloid GP 116

Of the 124 participants returning results for this trial, 67 (54.0%) submitted at least one reportable sequence variant in sample Myeloid GP 116 (please refer to the summary table on page 3 for details).

A detailed breakdown of nomenclature and educational aspects relating to variant interpretation will be subsequently published in a Myeloid Gene Panels 232401 report addendum to form the finalised trial report. Participants will be notified by email as soon as the final trial report is available. Please accept our apologies for any inconvenience caused.

Please note the recent release of v21.0.2 of the HGVS Nomenclature (dated 15 Mar 2024)⁵. The consistent use of standardised nomenclature with an appropriate reference sequence is critical for the effective communication of genetic testing results across the literature/databases and within a clinical setting. We strongly urge participants to comply with the latest HGVS recommendations for variant nomenclature and utilise transcript reference sequences designated by the MANE collaboration².

It is beyond the scope of this programme to comment conclusively on the clinical significance of the variants reported by participants. We acknowledge the limitations of this EQA exercise. The information provided herein is for participant information only. Clinical decision making with regards to variant interpretation, pathogenicity (driver status), actionability and predicted disease outcomes should not be based solely on comments provided by UK NEQAS LI.

Thank you to all participants who provided their full Laboratory Record information, as requested. The valuable methodological information supplied, including details regarding panel region of interest (ROI) and related reference sequences, facilitates an informative trial report.

Each financial year one Myeloid Gene Panels (Pilot – Not Accredited) trial distribution will focus on summarising the variants detected by participants (including methodological aspects) and the other will additionally provide educational elements related to variant biological classification and clinical interpretation. Please do contact us if you have any suggestions regarding how this pilot programme could be improved for future trial distributions: admin@ukneqasli.co.uk.

References

1. Li, MM *et al.* Standards and guidelines for the interpretation and reporting of sequence variants in cancer. *J Mol Diagn.* 19(1):4-23 (2017).
2. Morales, J *et al.* A joint NCBI and EMBL-EBI transcript set for clinical genomics and research. *Nature* 604:310–315 (2022).
3. Human Genome Variation Society (HGVS), <https://varnomen.hgvs.org/> (v20.05) – effective during the live trial period and accessed for analysis of trial Myeloid GP 232401.
4. Dunnen, JT *et al.* HGVS recommendations for the description of sequence variants: 2016 update. *Hum Mutat.* 37(6):564-9 (2016).
5. Human Genome Variation Society (HGVS), <https://hgvs-nomenclature.org/stable/> (v21.0.2) – accessed March 2024.

Information with respect to compliance with standards BS EN ISO/IEC 17043:2010

4.8.2 a) The proficiency testing provider for this programme is:

UK NEQAS for Leucocyte Immunophenotyping
Pegasus House, 4th Floor Suite
463A Glossop Road
Sheffield, S10 2QD
United Kingdom
Tel: +44 (0) 114 267 3600
e-mail: amanda.newbould@ukneqasli.co.uk

4.8.2 b) The coordinators of UK NEQAS LI programmes are Mr Liam Whitby (Director) and Mr Stuart Scott (Centre Manager).

4.8.2 c) Person(s) authorizing this report:

Mr Liam Whitby (Director) or Mr Stuart Scott (Centre Manager) of UK NEQAS LI.

4.8.2 d) Pre issue testing of samples for this programme is subcontracted, although the final decision about sample suitability lies with the EQA provider; no other activities in relation to this EQA exercise were subcontracted. Where subcontracting occurs, it is placed with a competent subcontractor and the EQA provider is responsible for this work.

4.8.2 g) The UK NEQAS LI Confidentiality Policy can be found in the Quality Manual which is available by contacting the UK NEQAS LI office. Participant details, their results and their performance data remain confidential unless revealed to the relevant NQAAP when a UK participant is identified as having performance issues.

4.8.2 i) All EQA samples are prepared in accordance with strict Standard Operational Procedures by trained personnel proven to ensure homogeneity and stability. Where appropriate/possible EQA samples are tested prior to issue. Where the sample(s) issued is stabilised blood or platelets, pre and post stability testing will have proved sample suitability prior to issue.

4.8.2 l), n), o), r) & s) Please refer to the UK NEQAS LI website at www.ukneqasli.co.uk for detailed information on each programme including the scoring systems applied to assess performance (for BS EN ISO/IEC 17043:2010 accredited programmes only). Where a scoring system refers to the 'consensus result' this means the result reported by the majority of participants for that trial issue. Advice on the interpretation of statistical analyses and the criteria on which performance is measured is also given. Please note that where different methods/procedures are used by different groups of participants these may be displayed within your report, but the same scoring system is applied to all participants irrespective of method/procedure used.

4.8.2 m) We do not assign values against reference materials or calibrants.

4.8.2 q) Details of the programme designs as authorized by The Steering Committee and Specialist Advisory Group can be found on our website at www.ukneqasli.co.uk. The proposed trial issue schedule for each programme is also available.

4.8.2 t) If you would like to discuss the outcomes of this trial issue, please contact UK NEQAS LI using the contact details provided. Alternatively, if you are unhappy with your performance classification for this trial, please find the appeals procedure at www.ukneqasli.co.uk/contact-us/appeals-and-complaints/

4.8.4) The UK NEQAS LI Policy for the Use of Reports by Individuals and Organisations states that all EQA reports are subject to copyright, and, as such, permission must be sought from UK NEQAS LI for the use of any data and/or reports in any media prior to use. See associated policy on the UK NEQAS LI website: <http://www.ukneqasli.co.uk/eqa-pt-programmes/new-participant-information/>

Pilot Myeloid Gene Panels (Not Accredited) 232401 – ADDENDUM

IMPORTANT: This addendum together with your individual participant report v1.0.1 (issued 18 September 2024) forms the complete and authorised Pilot Myeloid Gene Panels (Not Accredited) 232401 trial report for sample Myeloid GP 116.

The educational comments in this addendum (pages i-vii) are intended to supplement the data presented in the individual participant report (v1.0.1) dated 18 September 2024 (pages 1-11). It is beyond the scope of this programme to comment conclusively on the clinical significance of the variants reported by participants. We acknowledge the limitations of this EQA exercise. The information provided herein is for participant information only. Clinical decision making with regards to variant interpretation, pathogenicity/oncogenicity (driver status), actionability and predicted disease outcomes should not be based solely on comments collated by UK NEQAS LI.

Thank you to all participants who provided their full Laboratory Record information, as requested. We understand the data entry process for this programme can be laborious. However, the valuable methodological information supplied, including details regarding panel region of interest (ROI) and related reference sequences, facilitates an informative trial report.

Please do contact us if you have any suggestions regarding how this pilot programme could be improved for future trial distributions: admin@ukneqasli.co.uk.

Sample Myeloid GP 116

Overall 61 returning participants reported detection of the c.610T>C p.(Ser204Pro) missense variant in exon 4 of the *MPL* gene.

The median variant allele frequency (VAF) (DNA based assays, all panels and platforms) reported for the NM_005373.3:c.610T>C p.(Ser204Pro) *MPL* variant was 11.8% with an interquartile range of 1.2% (n=61).

Nomenclature was in full agreement at the cDNA level, with universal reference to versions of the MANE Select transcript (NM_005373/ENST00000372470)¹⁻⁴. At the protein level, 10 laboratories failed to include parentheses to indicate the predicted nature of the amino acid change and three centres utilised the less preferred single letter amino acid code. A single participant provided a nomenclature description without the p. prefix¹⁻³.

The classification of the noncanonical *MPL* exon 4 variant was broadly spread across strong (n=22), potential (n=21) and unknown (n=18) clinical significance. A summary of the currently available evidence relevant to the biological interpretation of the c.610T>C p.(Ser204Pro) variant can be found on page ii. The outline is for educational purposes only and may not be exhaustive.

Variants in the *MPL* gene can be found in up to 10% of essential thrombocythaemia (ET) and primary myelofibrosis (PMF) cases but are rarely seen in polycythemia vera (PV)⁵⁻⁷. The WHO and ICC major diagnostic criteria include analysis of the *JAK2*, *CALR* and *MPL* genes for ET (and PMF) cases⁵⁻⁸.

Activating variants in the *MPL* gene (also known as CD110 or *TPOR* in the legacy literature) typically occur within exon 10, which encodes the intracellular juxtamembrane and transmembrane regions of the thrombopoietin receptor protein⁹⁻¹⁰.

ET is characterised by megakaryocyte hyperplasia and thrombocytosis. Thrombopoietin ligand and the *MPL* encoded receptor are a major regulator of megakaryopoiesis and platelet formation. Classical exon

10 canonical *MPL* driver variants (for example, p.Trp515Leu/Lys/other and p.Ser505Asn) render the receptor constitutively active, leading to persistent hyperactivation of the Janus kinase-signal transducer and activator of transcription (JAK/STAT) pathway. This results in clonal expansion of myeloid progenitor cells that no longer require cytokine activation¹¹⁻¹³.

Summary of current available evidence to assist in the biological interpretation of the consensus NM_005373.3:c.610T>C p.(Ser204Pro) *MPL* exon 4 missense variant (legacy nomenclature: S204P).

- Population global minor allele frequency (MAF) for the c.610T>C p.(Ser204Pro) *MPL* variant is <0.00001% with just a single reported allele on gnomAD¹⁴ (equivalent to dbSNP rs913913702)¹⁵.
- The variant appears in two PMF cases on COSMIC (COSM26072 legacy, COSV65247294)¹⁶. An additional nine p.Ser204Pro entries appear in GENIE (including 3 ET cases)¹⁷. Multiple cases of the p.Ser204Pro *MPL* variant identified in ET patients are reported in the literature¹⁸⁻²⁴.
- Different missense changes at the same codon are also noted in the somatic variant databases, for example p.Ser204Phe (includes MPN cases)^{16,17} and p.Ser204Thr (solid tumour case)¹⁷.
- The p.Ser204 residue is located within the fibronectin type III extracellular domain of the thrombopoietin receptor protein but it is not a particularly well conserved amino acid position. Of note, protein alignment (Alamut Visual Plus v1.10)²⁵ places a proline residue at position 204 in mouse, dog and horse orthologs.
- *In silico* protein prediction tools (including meta-predictors) must always be applied with caution, especially in the context of a gain of function (GOF) mechanism. For this *MPL* p.Ser204Pro substitution routine use of protein level computational methods are not advocated (due to the variant not being located in a functional region known to harbour well characterised activating driver variants).
- Alamut Visual Plus and SpliceAI indicate no aberrant influence on splicing for the c.610T>C variant²⁵⁻²⁶.
- Functional studies reported in the literature support a GOF mechanism for the atypical *MPL* p.Ser204Pro variant. However, activation appears to be weaker than that observed for canonical GOF (exon 10) *MPL* variants^{19,20}.
 - Thrombocytosis induced by *MPL* p.Ser204Pro does not appear to be due to a defect in cell surface localisation of the receptor¹⁹.
 - No constitutive activation of STAT3, STAT5 or AKT was observed in UT7 cells expressing *MPL* p.Ser204Pro when phosphorylation status was examined by Western blot¹⁹.
 - However, a more sensitive luciferase reporter assay approach indicated *MPL* p.Ser204Pro is a weak JAK-STAT signalling GOF variant capable of inducing constitutive ligand (TPO)-independent STAT activation^{19,20}. Both Cabagnols *et al.* and Milosevic Feenstra *et al.* independently remark that a longer period (post transfection of the JAK2-deficient gamma2A cells) was required for the *MPL* p.Ser204Pro variant to induce JAK2/STAT activation compared to the *MPL* canonical variant positive control^{19,20}.
 - Western blotting suggested a more prolonged ligand (TPO)-induced STAT phosphorylation for UT7 cells expressing *MPL* p.Ser204Pro compared to wildtype¹⁹.
 - Cabagnols *et al.* observed that the *MPL* p.Ser204Pro variant could induce cytokine-independent growth in UT7 cells but with low efficiency. *MPL* p.Ser204Pro UT7 cells were also more resistant than *MPL* wildtype cells to cytokine deprivation¹⁹.
 - Nevertheless, Milosevic Feenstra *et al.* reported that expression of *MPL* p.Ser204Pro in the interleukin-3 (IL-3) dependent murine cell line Ba/F3 did not confer cytokine-independent growth or hypersensitivity to IL-3 (in contrast to the *MPL* p.Trp515Lys positive control)²⁰.
 - Cabagnols *et al.* postulate that variants, such as *MPL* p.Ser204Pro, with lower oncogenic potential may need to associate with other driver variants to give rise to MPN. Of note, for

the two ET cases harbouring *MPL* p.Ser204Pro described in their study both featured at least one additional variant categorised by the authors as a driver (*ASXL1*) or potential driver (*HELZ*)¹⁹.

Of the 63 laboratories not returning the NM_005373.3:c.610T>C p.(Ser204Pro) *MPL* exon 4 missense variant, 92.1% (n=58) of participants provided methodological information via their Laboratory Record.

- A minority of centres (6.9%, 4/58) did not feature the *MPL* gene on their panel.
- 82.8% (48/58) laboratories included the *MPL* gene on their panel but exon 4 was not stated in their region of interest (ROI).
- A further 6.9% (4/58) of participants were known to feature the *MPL* gene on their panel but failed to provide unequivocal ROI information.
- The remaining two (3.4%) participants utilised the (IonTorrent) Oncomine Myeloid Research Assay and are expected to include *MPL* exon 4 in their local assay. No QC issues were declared at trial results submission. The median VAF=11.8% was higher than their stated minimum level for reporting identification of a single nucleotide substitution (1-3%). However, details regarding locally applied ROI was absent/limited and therefore, their failure to report the *MPL* p.(Ser204Pro) variant remains an equivocal false negative result.

Eight laboratories reported the c.1211G>A p.(Cys404Tyr) missense change in the *CBL* gene.

Variants in this gene have been previously reported in ET patients²⁷⁻²⁸. Given its presence at a low level (median VAF = 1%) the variant was reported by only a minority of returning participants and therefore, it will not be discussed further in this trial report.

Of note, one laboratory is suspected to have undertaken a sample transposition. The *MPL* gene does not feature in the stated scope of their in-house amplicon-based panel. However, the centre submitted out of consensus *ASXL1* (NM_015338.5:c.2831T>A p.(Leu944*)) and *TP53* (NM_000546.5:c.490A>T p.(Lys164*)) variants; both classified by the participant as of strong clinical significance.

Thank you to those laboratories responding to the additional question regarding which guideline(s)/ recommendation(s) are routinely utilised to classify variants detected during tumour testing undertaken primarily for the identification of somatic variants (in the context of myeloid neoplasms). The results are summarised below.

	n
Li MM <i>et al.</i> (2017) Standards and Guidelines for the Interpretation and Reporting of Sequence Variants in Cancer: A Joint Consensus Recommendation of the Association for Molecular Pathology, American Society of Clinical Oncology, and College of American Pathologists. <i>J Mol Diagn.</i> 19(1):4-23	82
Richards S <i>et al.</i> (2015) Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. <i>Genet Med.</i> 17(5):405-24 [germline]	46
Horak P <i>et al.</i> (2022) Standards for the classification of pathogenicity of somatic variants in cancer (oncogenicity): Joint recommendations of Clinical Genome Resource (ClinGen), Cancer Genomics Consortium (CGC), and Variant Interpretation for Cancer Consortium (VICC). <i>Genet Med.</i> 24(5):986-998. Erratum in: <i>Genet Med.</i> 24(9):1991	28
Froyen G <i>et al.</i> (2019) Standardization of somatic variant classifications in solid and haematological tumours by a two-level approach of biological and clinical Classes: An initiative of the Belgian ComPerMed Expert Panel. <i>Cancers (Basel)</i> 11(12):2030	17
Koeppel F <i>et al.</i> (2021) Standardisation of pathogenicity classification for somatic alterations in solid tumours and haematologic malignancies. <i>Eur J Cancer.</i> 159:1-15	8
Sukhai MA <i>et al.</i> (2016) A classification system for clinical relevance of somatic variants identified in molecular profiling of cancer. <i>Genet Med.</i> 18(2):128-36	4

Conclusions

Driver variants identified in *JAK2* (p.Val617Phe, exon 12), *CALR* (exon 9) and *MPL* (exon 10) during standard diagnostic analysis account for >90% of classical *BCR::ABL1* negative MPN cases⁵⁻⁹. However, in approximately 10-20% of ET patients the genetic driver(s) of clonality remain unknown²⁴. Such cases are members of the 'triple-negative' MPN subgroup which are frequently discussed in the literature.

For sample Myeloid GP 116 (case with a working diagnosis of ET), the exon 4 location of the *MPL* c.610T>C p.(Ser204Pro) variant rendered it beyond the scope of many participating laboratories' NGS gene panel assays. For those laboratories successfully identifying the variant, its position outside of the established exon 10 'hotspot' regions and the published functional studies (indicating a weaker GOF compared to canonical variant controls) are likely to have contributed to the spread of classification for this atypical missense change.

For this trial, at least 44.4% (48/108) of participants featuring the *MPL* gene on their NGS panel did not encompass exon 4 within their stated region of interest (most of these laboratories featured only exon 10 in their panel). The expansion of sequencing to include the analysis of all coding exons of *MPL* is pertinent for those patients considered to represent a triple-negative MPN case. Current guidelines recommend the use of analytically sensitive assays at diagnosis (specifically 1-3% limit of detection for *MPL*) and, for MPN triple-negative cases, the consideration of noncanonical *MPL* (and *JAK2*) variants is highlighted⁷⁻⁹. Maddali *et al.* note 10-20% of patients with triple-negative ET are found to harbour

atypical variants (noncanonical mutations) in *MPL* and *JAK2*²⁷, some of which will be of uncertain significance. Of note, variant detection rates can be influenced by the diagnostic stringency of a study cohort. The aetiology of triple-negative thrombocytosis is heterogeneous and many presenting ?ET patients will lack identification of a driver variant on comprehensive NGS extended gene panel testing^{9,21}. On occasion, activating *MPL/JAK2* variants can be germline inherited and therefore indicative of hereditary thrombocytosis rather than ET²⁹⁻³⁰.

Although bone marrow histology remains key to diagnose triple-negative ET/MF, the application of broader NGS gene panels and whole exome sequencing (WES) is contributing to the characterisation of these disorders by identifying variants in (putative) driver genes for a sub-set of patients. Such information is increasingly available for use in clinical decision-making. In addition to noncanonical *JAK2* and *MPL* variants, sequence changes of clinical interest have been reported in various genes including those encoding epigenetic modifiers (*ASXL1*, *TET2*, *DNMT3A*, *IDH1*, *IDH2*) the spliceosome machinery (*SF3B1*, *SRSF2*) and regulators of cytokine signalling (*CBL*, *SH2B3*)^{7,9,10,22,24,27,28}. The natural history of the *BCR::ABL1* negative MPNs can be marked by the acquisition of additional variants over long periods of time, with certain variants thought to be involved in disease initiation and others secondary evolution^{10,31,32}.

In general, triple-negative ET patients demonstrate a favourable prognosis (indolent disease with a low incidence of vascular events). Interestingly, triple-negative PMF status is associated with poorer survival (aggressive myeloid neoplasm with a higher risk of leukemic evolution)⁵. The genomic characterisation of patients offers the possibility for personalised diagnosis, risk stratification and targeted treatment. Traditionally MPN prognosis has mainly been informed by clinical and haematologic parameters. However, risk stratification models incorporating extended gene analysis have been developed with the aim of better estimating overall survival and targeting therapy^{7,10}, including in the specific context of ET^{33,34}.

It is important to be mindful that a large proportion of suspected MPN patients have been found not to carry additional variants of biological significance beyond a *JAK2*, *CALR* or *MPL* driver variant when analysed via NGS targeted panels, WES or whole genome sequencing (WGS)^{10,21}. However, for blast phase MPN patients with a canonical *JAK2*, *CALR* or *MPL* driver variant identified at initial diagnosis, follow-up analysis by a broader NGS gene panel may add information regarding prognosis, eligibility for clinical trials and targeted treatment^{9,35}.

Duncavage *et al.* have produced a helpful guide to the application of genomic profiling for clinical decision making in MPN and other myeloid neoplasms³⁶ but it is accepted that this is an ever developing field.

Godfrey *et al.* have very recently published a short report regarding the clinical utility of investigations in triple-negative thrombocytosis specifically³⁷. The authors report the variation in investigative practice between centres, including differences in myeloid NGS gene panel scope. They also note a low incidence of noncanonical driver variants detected by extended gene panel analysis. In this study the final clinical diagnosis of MPN was most strongly predicted by histology rather than extended gene screening in their triple-negative cohort (with broad inclusion criteria).

This trial represents the occurrence of a noncanonical *MPL* variant in a suspected MPN triple-negative patient. The exercise raises the importance of a thorough appraisal of available evidence during the variant classification process and draws attention to the contribution of published functional studies to assist in the interpretation of biological significance for atypical variants. While the application of extended NGS gene panel testing has greatly contributed to the characterisation MPNs; at the time of writing, challenges remain regarding the actionability of such noncanonical variants in *MPL* (and other genes) in the clinic.

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