Utility of clinical comprehensive genomic characterization for diagnostic categorization in patients presenting with hypocellular bone marrow failure syndromes

Piers Blombery,1,2,3 Lucy C. Fox,3,4,5* Georgina L. Ryland,3* Ella R. Thompson,2,3 Jennifer Lickiss,3 Michelle McBean,3 Satwica Yerneni,3 David Hughes,5 Anthea Greenway,6 Francoise Mechinaud,6 Erica M. Wood,5 Graham J. Lieschke,5,7 Jeff Szer,1 Pasquale Barbaro,8 John Roy,9 Joel Wight,9 Elly Lynch,10,11,12 Melissa Martyn,10,11,12 Clara Gaff10,11,12 and David Ritchie1

1Clinical Hematology, Peter MacCallum Cancer Center/Royal Melbourne Hospital, Melbourne, Victoria; 2University of Melbourne, Melbourne, Victoria; 3Department of Pathology, Peter MacCallum Cancer Center, Melbourne, Victoria; 4Epworth Healthcare, Melbourne, Victoria; 5Transfusion Research Unit, School of Public Health & Preventive Medicine, Monash University, Melbourne, Victoria; 6Royal Children’s Hospital, Melbourne, Victoria; 7Australian Regenerative Medicine Institute, Monash University, Melbourne, Victoria; 8Children’s Health Queensland and University of Queensland, South Brisbane, Queensland; 9Department of Hematology, Austin Health, Melbourne, Victoria; 10Melbourne Genomics Health Alliance, Melbourne, Victoria; 11Victorian Clinical Genetics Service, Melbourne, Victoria and 12Murdoch Children’s Research Institute, Melbourne, Victoria, Australia

*LCF and GLR contributed equally as co-second authors

©2021 Ferrata Storti Foundation. This is an open-access paper. doi:10.3324/haematol.2019.237693

Received: September 6, 2019.
Accepted: February 7, 2020.
Correspondence: PETERS BLOMBERY - piers.blombery@petermac.org
Supplementary Material

Utility of clinical comprehensive genomic characterisation for diagnostic categorisation in patients presenting with hypocellular bone marrow failure syndromes

Piers Blombery¹,²,³, Lucy Fox²,⁴,⁵*, Georgina L. Ryland³*, Ella R. Thompson²,³, Jennifer Lickiss³, Michelle McBean³, Satwica Yerneni³, David Hughes⁶, Anthea Greenway⁶, Francoise Mechinaud⁶, Erica M. Wood⁵, Graham J. Lieschke¹,⁷, Jeff Szer¹, Pasquale Barbaro⁸, John Roy⁹, Joel Wight⁹, Ella Lynch¹⁰,¹¹,¹², Melissa Martyn¹⁰,¹¹,¹², Clara Gaff¹,¹⁰,¹², David Ritchie¹

¹Clinical Haematology, Peter MacCallum Cancer Centre/Royal Melbourne Hospital, Melbourne, Australia
²University of Melbourne, Melbourne, Australia
³Department of Pathology, Peter MacCallum Cancer Centre, Melbourne, Australia
⁴Epworth Pathology, Melbourne, Australia
⁵Transfusion Research Unit, School of Public Health & Preventive Medicine, Monash University, Melbourne, Australia
⁶Royal Children’s Hospital, Melbourne, Australia
⁷Australian Regenerative Medicine Institute, Monash University
⁸Children’s Health Queensland and University of Queensland
⁹Department of Haematology, Austin Health, Melbourne, Australia
¹⁰Melbourne Genomics Health Alliance
¹¹Victorian Clinical Genetics Service, Melbourne Australia
¹²Murdoch Children’s Research Institute, Melbourne Australia

*these authors contributed equally to this work

Supplementary Methods

Study design

This was a prospective study of patients presenting with bone marrow failure (BMF) through the Peter MacCallum Cancer Centre, The Royal Melbourne Hospital, Royal Children’s Hospital and Austin Hospital, Victoria, Australia. Eligibility criteria were (i) age ≥3 months (ii) a clinicopathological diagnosis of either acquired aplastic anaemia (aAA), an inherited/genetic BMF syndrome (IBMFS), hypoplastic myelodysplastic syndrome (hMDS) or a BMF syndrome characterised by marrow hypoplasia/aplasia but not able to be definitively categorised. All participants were under ongoing investigation into the cause of their BMF. Patients (or their guardians) provided written informed consent after pre-test counselling and assessment. Research was conducted after institutional review board ethics approval.
Targeted sequencing

Patient samples including peripheral blood, bone marrow aspirate, buccal swab, skin biopsy and cultured skin fibroblasts were analysed using three NGS-based sequencing panels, as described below.

**BMF panel**

DNA (300 ng) was sheared by focused acoustic sonication (Covaris, MA, USA) and libraries prepared using the KAPA Hyper Prep Kit (KAPA Biosystems, MA, USA), followed by enrichment using a custom SureSelectXT hybridisation-based capture panel (Agilent, CA, USA) and sequenced on an Illumina NextSeq500 (Illumina, CA, USA) with 75 bp paired-end reads. After base calling and de-multiplexing, a Seqliner-framework analysis pipeline was used to align reads to the human reference genome (GRCh37 assembly) using BWA-MEM, followed by marking of duplicate reads, base quality score recalibration, local indel realignment and germline variant calling using GATK Haplotype Caller. In addition the targeted panel aligned sequence data was processed through a dedicated bioinformatics pipeline which included variant calling with GATK4/Mutect2 (https://software.broadinstitute.org/gatk/) in order to improve detection of low level acquired variants. VCFs were analysed in PathOS including variants occurring within the coding regions and flanking splice sites (within 2 bp) of the 85 genes and transcripts listed in Supplementary Table 1. The BMF panel includes genes implicated in both IBMFS and aAA. Acquired variants were curated for pathogenicity as above. Germline variants were classified according to the American College of Medical Genetics and Genomics standards for interpretation of sequence variants and reviewed at a series of multidisciplinary team meetings. Pathogenic and likely pathogenic variants of clinical significance were confirmed by an alternative method. Only pathogenic and likely pathogenic variants are included in the description of this cohort. Some variants were also investigated in parental samples to establish phase and segregation. Genome-wide copy number analysis was performed by comparing read counts from on and off target reads to a pooled reference to correct for enrichment and sequencing biases.

**Whole exome sequencing**

SureSelectQXT (Agilent) libraries were prepared from 50 ng DNA followed by targeted enrichment using the Clinical Research Exome V1 (Agilent) and sequenced on an Illumina HiSeq 4000 (Illumina) with 150 bp paired-end reads. A Seqliner-framework analysis pipeline and germline variant curation in PathOS was performed as described above. ANKRD26, DNAJC21, ETV6, SAMD9, SAMD9L, SMARCD2, SRP54 and THPO were assessed in all patients in addition to all genes present in the targeted panel (93 genes listed in Supplementary Table 1). If no causative variant was identified, whole exome sequencing (WES) analysis was extended to include additional genes based on the patient’s clinical phenotype.

**RNAseq**

Samples for whole transcriptome sequencing were prepared using the TruSeq RNA Sample Preparation Kit (Illumina) from 1 μg total RNA according to standard protocols. Samples were sequenced on an Illumina NextSeq500 (Illumina) with 75 bp paired-end reads. Alignment was
performed using HISAT2 followed by generation of read counts using HTSeq. Quantile normalized counts were generated using Limma.

**Droplet Digital PCR**

Droplet digital PCR (ddPCR) analysis was performed using the Bio-Rad Droplet Digital PCR system (Bio-Rad, CA, USA) following manufacturer’s protocols. An allele-specific PCR assay to detect and quantify the fractional abundance of the RUNX1 c.496C>T variant and corresponding wildtype allele was custom designed using PrimePCR (BioRad). Details of the TERT c.-124C>T ddPCR assay have been described previously\(^3\). ddPCR was performed using approximately 50 ng DNA per reaction on the QX200 Droplet Digital PCR System and data were analysed using QuantaSoft software, to a detection limit of 0.2%.

**Supplementary Table 1. BMF genes assessed by panel and whole exome sequencing.**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transcript</th>
<th>Targeted exons</th>
<th>Gene</th>
<th>Transcript</th>
<th>Targeted exons</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACD</td>
<td>NM_001082486.1</td>
<td>All coding</td>
<td>MPL</td>
<td>NM_005373.2</td>
<td>All coding</td>
</tr>
<tr>
<td>AK2</td>
<td>NM_013411.4</td>
<td>All coding</td>
<td>NHP2</td>
<td>NM_017838.3</td>
<td>All coding</td>
</tr>
<tr>
<td>ANKRD26*</td>
<td>NM_014915.2</td>
<td>All coding &amp; 5’UTR</td>
<td>NOP10</td>
<td>NM_018648.3</td>
<td>All coding</td>
</tr>
<tr>
<td>ASXL1</td>
<td>NM_015338.5</td>
<td>12</td>
<td>NRAS</td>
<td>NM_002524.4</td>
<td>2-4</td>
</tr>
<tr>
<td>ATR</td>
<td>NM_001184.3</td>
<td>All coding</td>
<td>PALB2</td>
<td>NM_024675.3</td>
<td>All coding</td>
</tr>
<tr>
<td>BCO1</td>
<td>NM_017745.5</td>
<td>All coding</td>
<td>PARN</td>
<td>NM_002582.3</td>
<td>All coding</td>
</tr>
<tr>
<td>BCO1L1</td>
<td>NM_021946.4</td>
<td>All coding</td>
<td>PIGA</td>
<td>NM_002641.3</td>
<td>All coding</td>
</tr>
<tr>
<td>BRCA2</td>
<td>NM_000059.3</td>
<td>All coding</td>
<td>RAD51C</td>
<td>NM_058216.2</td>
<td>All coding</td>
</tr>
<tr>
<td>BRIP1</td>
<td>NM_032043.2</td>
<td>All coding</td>
<td>RBM8A</td>
<td>NM_005105.4</td>
<td>All coding</td>
</tr>
<tr>
<td>CALR</td>
<td>NM_004343.3</td>
<td>9 (AA352-400)</td>
<td>RPL11</td>
<td>NM_000975.3</td>
<td>All coding</td>
</tr>
<tr>
<td>CBL</td>
<td>NM_005188.3</td>
<td>All coding</td>
<td>RPL15</td>
<td>NM_002948.3</td>
<td>All coding</td>
</tr>
<tr>
<td>CSF3R</td>
<td>NM_156039.3</td>
<td>All coding</td>
<td>RPL26</td>
<td>NM_000987.3</td>
<td>All coding</td>
</tr>
<tr>
<td>CSM1D</td>
<td>NM_033225.5</td>
<td>All coding</td>
<td>RPL35A</td>
<td>NM_000996.2</td>
<td>All coding</td>
</tr>
<tr>
<td>CTC1</td>
<td>NM_025099.5</td>
<td>All coding</td>
<td>RPL5</td>
<td>NM_000969.3</td>
<td>All coding</td>
</tr>
<tr>
<td>DDX41</td>
<td>NM_016222.2</td>
<td>All coding</td>
<td>RPS10</td>
<td>NM_001014.4</td>
<td>All coding</td>
</tr>
<tr>
<td>DKC1</td>
<td>NM_001363.3</td>
<td>All coding</td>
<td>RPS19</td>
<td>NM_001022.3</td>
<td>All coding</td>
</tr>
<tr>
<td>DNAJC21*</td>
<td>NM_194283.3</td>
<td>All coding</td>
<td>RPS24</td>
<td>NM_033022.3</td>
<td>All coding</td>
</tr>
<tr>
<td>DNM3T3A</td>
<td>NM_022552.4</td>
<td>All coding</td>
<td>RPS26</td>
<td>NM_001029.3</td>
<td>All coding</td>
</tr>
<tr>
<td>ELANE</td>
<td>NM_001972.2</td>
<td>All coding</td>
<td>RPS29</td>
<td>NM_001032.4</td>
<td>All coding</td>
</tr>
<tr>
<td>ERBB2</td>
<td>NM_004448.2</td>
<td>8, 17, 19-21, 27</td>
<td>RPS7</td>
<td>NM_001011.3</td>
<td>All coding</td>
</tr>
<tr>
<td>ERCC4</td>
<td>NM_005236.2</td>
<td>All coding</td>
<td>RTEL1</td>
<td>NM_032957.4</td>
<td>All coding</td>
</tr>
<tr>
<td>ERCC6L2</td>
<td>NM_001010895.2</td>
<td>All coding</td>
<td>RUNX1</td>
<td>NM_001754.4</td>
<td>All coding</td>
</tr>
<tr>
<td>ETV6*</td>
<td>NM_001987.4</td>
<td>All coding</td>
<td>SAMD9*</td>
<td>NM_017654.3</td>
<td>All coding</td>
</tr>
<tr>
<td>EZH2</td>
<td>NM_00456.4</td>
<td>All coding</td>
<td>SAMD9L*</td>
<td>NM_152703.2</td>
<td>All coding</td>
</tr>
<tr>
<td>FANCA</td>
<td>NM_000135.2</td>
<td>All coding</td>
<td>SBDS</td>
<td>NM_016038.2</td>
<td>All coding</td>
</tr>
<tr>
<td>FANCB</td>
<td>NM_001018113.1</td>
<td>All coding</td>
<td>SETBP1</td>
<td>NM_015559.2</td>
<td>4</td>
</tr>
<tr>
<td>FANCC</td>
<td>NM_000136.2</td>
<td>All coding</td>
<td>SF3B1</td>
<td>NM_012433.2</td>
<td>14-18</td>
</tr>
<tr>
<td>FANCD2</td>
<td>NM_001018115.1</td>
<td>All coding</td>
<td>SH2D1A</td>
<td>NM_002351.4</td>
<td>All coding</td>
</tr>
<tr>
<td>FANCE</td>
<td>NM_021922.2</td>
<td>All coding</td>
<td>SLX4</td>
<td>NM_032444.2</td>
<td>All coding</td>
</tr>
<tr>
<td>FANCF</td>
<td>NM_022725.3</td>
<td>All coding</td>
<td>SMARCA1</td>
<td>NM_014140.3</td>
<td>All coding</td>
</tr>
<tr>
<td>FANCIG</td>
<td>NM_004629.1</td>
<td>All coding</td>
<td>SMARCD2*</td>
<td>NM_001098426.1</td>
<td>All coding</td>
</tr>
<tr>
<td>FANCJ</td>
<td>NM_00113378.1</td>
<td>All coding</td>
<td>SRP54*</td>
<td>NM_003136.3</td>
<td>All coding</td>
</tr>
<tr>
<td>FANCL</td>
<td>NM_018062.3</td>
<td>All coding</td>
<td>SRP72</td>
<td>NM_006947.3</td>
<td>All coding</td>
</tr>
<tr>
<td>FANCN</td>
<td>NM_020937.2</td>
<td>All coding</td>
<td>SRSF2</td>
<td>NM_003016.4</td>
<td>1</td>
</tr>
<tr>
<td>G6PC3</td>
<td>NM_138387.3</td>
<td>All coding</td>
<td>TCIRG1</td>
<td>NM_006019.3</td>
<td>All coding</td>
</tr>
<tr>
<td>GATA1</td>
<td>NM_002049.3</td>
<td>All coding</td>
<td>TERT</td>
<td>NM_198253.2</td>
<td>All coding</td>
</tr>
<tr>
<td>GATA2</td>
<td>NM_032638.4</td>
<td>All coding</td>
<td>TET2</td>
<td>NM_001127208.2</td>
<td>All coding</td>
</tr>
</tbody>
</table>

\(^3\) ORI16 and ORI17 and KMT2A c.405C>T ddPCR assays have been described previously. ddPCR was performed using approximately 50 ng DNA per reaction on the QX200 Droplet Digital PCR System and data were analysed using QuantaSoft software, to a detection limit of 0.2%.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Reference</th>
<th>Coding Region</th>
<th>Gene</th>
<th>Reference</th>
<th>Coding Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>GFI1</td>
<td>NM_005263.3</td>
<td>All coding</td>
<td>THPO*</td>
<td>NM_000460.2</td>
<td>All coding</td>
</tr>
<tr>
<td>HAX1</td>
<td>NM_006118.3</td>
<td>All coding</td>
<td>TINF2</td>
<td>NM_001099274.1</td>
<td>All coding</td>
</tr>
<tr>
<td>IDH1</td>
<td>NM_005896.2</td>
<td>4</td>
<td>TP53</td>
<td>NM_000546.5</td>
<td>All coding</td>
</tr>
<tr>
<td>IDH2</td>
<td>NM_002168.2</td>
<td>4</td>
<td>U2AF1</td>
<td>NM_006758.2</td>
<td>2, 6</td>
</tr>
<tr>
<td>IKZF1</td>
<td>NM_006060.4</td>
<td>All coding</td>
<td>VPS45</td>
<td>NM_007259.4</td>
<td>All coding</td>
</tr>
<tr>
<td>JAGN1</td>
<td>NM_032492.3</td>
<td>All coding</td>
<td>WAS</td>
<td>NM_000377.2</td>
<td>All coding</td>
</tr>
<tr>
<td>JAK2</td>
<td>NM_004972.3</td>
<td>All coding</td>
<td>WRAP53</td>
<td>NM_018081.2</td>
<td>All coding</td>
</tr>
<tr>
<td>JAK3</td>
<td>NM_000215.3</td>
<td>11, 15, 16</td>
<td>WT1</td>
<td>NM_024426.4</td>
<td>7-9</td>
</tr>
<tr>
<td>KIT</td>
<td>NM_000222.2</td>
<td>All coding</td>
<td>ZRSR2</td>
<td>NM_005089.3</td>
<td>All coding</td>
</tr>
<tr>
<td>KRAS</td>
<td>NM_033360.2</td>
<td>2-4</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Gene analysed by WES only
AA, amino acid.
**Supplementary Clinical Data**

**Patient ID #4:** A 5-year-old male presented shortly after birth with anaemia and thrombocytopenia requiring transfusion support. Bone marrow biopsy at 11 weeks of age demonstrated mild to moderate dyserythropoiesis and dysgranulopoiesis but insufficient morphological features for a formal diagnosis of a myelodysplastic syndrome. Metaphase cytogenetic analysis and FISH for chromosomes 5, 7 and 8 was normal. A nonsense variant in \textit{RUNXI} [NM_001754.4:c.496C>T; p.(Arg166*)] was detected with targeted sequencing at 4.8% variant allele frequency. This variant was quantitatively monitored in the bone marrow over time by allele specific ddPCR and was noted to be steadily increasing and reaching 11.8% over a 15 month period (Supp Fig 1). The variant was not detected in a skin biopsy sample. The patient underwent a matched unrelated donor allogeneic bone marrow transplant with subsequent resolution of cytopenias.

![Supplementary Figure 1. Quantitation of RUNXI c.496C>T by droplet digital PCR over time in a 5-year-old with refractory cytopenia of childhood.](image-url)

**Patient ID #13:** A 6-month-old female was noted to be severely anaemic at birth (haemoglobin 50 g/L) and experienced bilateral watershed cerebral infarcts, respiratory failure requiring intubation and anuria. A clinical diagnosis was unable to be made. BMF panel testing on bone marrow aspirate identified a heterozygous missense variant in \textit{RPS19} [NM_001022.3:c.184C>T; p.(Arg62Trp)] providing a diagnosis of Diamond-Blackfan anaemia. The \textit{RPS19} Arg62Trp has been described in multiple families as both a \textit{de novo} and an inherited variant segregating with Diamond-Blackfan anaemia with an autosomal dominant mode of inheritance\textsuperscript{4-6}. The patient had significant clinical improvement upon introduction of steroids and resolution of the profound anaemia, and continues on low-dose prednisolone daily.
**Patient ID #14:** A 4-year-old male was noted to have incidentally identified anaemia aged 12 months. A clinicopathological diagnosis of Diamond-Blackfan anaemia was made based on bone marrow aspirate and trephine demonstrating severe red cell hypoplasia in the setting of gradual onset anaemia and marked macrocytosis. No genetic testing was performed at the time of this clinical diagnosis. BMF panel testing identified a frameshift variant in *RPS19* [NM_001022.3:c.251_252del; p.(Arg84Lysfs*69)]. Parental testing confirmed the *de novo* origin of this variant. This variant has previously been described in a patient with Diamond-Blackfan anaemia 5.

**Patient ID #20:** A 5-year-old female had dysmorphic features including squared and dysplastic ears with hypoplastic lobes, and scaphocephaly. She was born pre-term (36 weeks gestation) requiring ventilation support and admission for sepsis, thrombocytopenia and mild anaemia. At 3 months of age she was diagnosed with CMV colitis resulting in a sigmoid stricture and a large bowel obstruction that required surgical correction and nasogastric tube feeding until 2 years of age. There was no family history of an IBMFS. Bone marrow biopsy performed shortly after birth was normocellular with subtle trilineage dysplasia, and monosomy 7 was detected by metaphase cytogenetics and FISH. Repeat biopsy at 2-3 month intervals consistently detected the monosomy 7 by FISH but at reducing frequency until becoming undetectable over a period of approximately 32 months. Trilineage dysplasia remained stable over this time period, with some minor improvement noted at the time of monosomy 7 resolution. WES performed on a stored bone marrow aspirate sample (collected 5 months after birth) identified a NM_017654.3:c.4057A>G; p.(Lys1353Glu) missense variant in *SAMD9* at approximately 30% variant allele frequency, along with the presence of monosomy 7 (Supp Fig 2A & B). Testing of DNA from skin fibroblasts from the patient and her parents confirmed a *germline de novo* origin of this variant. The Lys1353Glu has not been reported in population databases or in clinical case series of patients with bone marrow failure.
Supplementary Figure 2. (A) SAMD9 Lys1353Glu detected in bone marrow and skin fibroblast samples from a 5-year-old with BMF. This variant was not detected in DNA from either parent. (B) Monosomy 7 detected in a bone marrow sample collected 5 months after birth.

**Patient ID #23:** A 7-year-old male was incidentally discovered to have monosomy 7 by SNP array while being investigated for autism spectrum disorder at 2 years of age. There was no history of BMF in the family. A bone marrow biopsy was hypocellular with trilineage dysplasia and no blast excess. WES identified a previously unreported missense variant in SAMD9 [NM_017654.3:c.2318T>C; p.(Ile773Thr)] which was confirmed to be de novo through parental testing. The patient was treated with 6 cycles of azacitidine. The monosomy 7 clone reduced from 86% to 19% measured by FISH over this 6 month time period however whether this was due to azacitidine or spontaneous resolution (as is occasionally observed in SAMD9 and related abnormalities) cannot be definitively determined. The patient underwent an unrelated cord blood transplant and is in an ongoing complete response with full donor chimerism.

**Patient ID #25:** A 14-year-old female presented at birth with severe anaemia and neutropenia. There was no family history of an IBMFS. Bone marrow biopsy was normocellular with markedly reduced erythropoiesis and myeloid maturation arrest. The patient was not responsive to corticosteroids and remained transfusion-dependent with persistent neutropenia and recurrent infections. A
heterozygous deletion on chromosome 3 involving RPL35A was detected by BMF panel copy number analysis (Supp Fig 3). Subsequent copy number analysis by SNP array confirmed a terminal deletion of approximately 0.5 Mb on the long arm of one chromosome 3. In addition, the long arm of chromosome 3 showed an apparently acquired genotyping profile consistent with progressive copy number neutral loss of heterozygosity increasing from 10% near the centromere to 30% towards the telomere, similar to that previously described in two siblings with Diamond-Blackfan anaemia and a RPL35A deletion.

**Supplementary Figure 3.** Copy number analysis from BMF panel showing heterozygous deletion of RPL35A.

**Patient ID #31:** A 16-year-old male presented at 11 weeks of age with severe anaemia. There were no dysmorphic features or developmental delay and no family history of an IBMFS. A bone marrow biopsy demonstrated marked erythroid hypoplasia. The anaemia responded to corticosteroid treatment however the patient remained steroid-dependent until approximately 10 years of age when he was successfully weaned off. Copy number analysis identified a heterozygous deletion of exons 2 and 3 in RPS19 (Supp Fig 4).

**Supplementary Figure 4.** Copy number analysis from BMF panel showing heterozygous deletion of RPS19 exons 2-3.

**Patient ID #32:** A 5-year-old female had presented as an infant with cellulitis and was noted to be neutropenic. Both her mother and maternal grandmother had also experienced lifelong severe neutropenia, but generally experienced good health (with occasional hospital admissions for infection only) and did not require G-CSF therapy regularly. The child was developmentally normal with no additional medical issues. WES detected a heterozygous in-frame deletion in SRP54 [NM_003136.3:c.349_351del; p.(Thr117del)]. The Thr117del is the most commonly described variant in SRP54 in case series of severe congenital neutropenia to date and has been described as both a de novo and an inherited variant segregating with an autosomal dominant mode of inheritance. The
SRP54 Thr117del was subsequently detected in both her mother and grandmother, providing a genetic diagnosis to three generations of a family who had experienced uncharacterised BMF for over 50 years.

**Patient ID #39:** A 27-year-old otherwise well female presented with pancytopenia. No congenital abnormalities were present on physical exam. Bone marrow aspirate and trephine demonstrated marked hypocellularity. Eltrombopag was commenced for presumed aplastic anaemia without improvement in blood counts. There was no family history of an IBMFS, however consanguinity was noted. SNP array testing on a peripheral blood sample demonstrated long continuous stretches of homozygosity (>5Mb) on chromosomes 4, 6, 7, 16, 19 and 21 representing approximately 6% of the genome. BMF panel testing on bone marrow aspirate demonstrated a homozygous FANCA missense variant [NM_000135.2:c.2980A>G; p.(Ser994Gly)]. This variant occurs within the conserved splice sequence motif of the exon 30/intron 30 splice donor site and RNAseq analysis from unselected bone marrow cells showed skipping of exon 30 (Supp Fig 5). Chromosomal breakage studies (using diepoxybutane) performed on peripheral blood as well as cultured skin fibroblasts were both equivocal with evidence of increased chromosomal break relative to negative control but insufficient for a positive result. However, absence of monoubiquitination of FANCD2 in PHA-stimulated blood cells on western blot was observed consistent with inactivation of FANCA and a diagnosis of Fanconi anaemia.

**Supplementary Figure 5.** RNAseq from bone marrow cells showing exon 30 skipping in FANCA as a result of NM_000135.2: c.2980A>G; p.(Ser994Gly).
Patient ID #44: A 35-year-old male presented at birth with tetralogy of Fallot and atrioventricular canal defect requiring multiple cardiothoracic operations. He was of short stature with a webbed neck, dysmorphic facies and earlobes, and lacked thumb bones bilaterally. Other medical issues included a solitary kidney, obstructive sleep apnoea, type two diabetes mellitus, scoliosis with restrictive lung disease and learning difficulties. His sibling had died at age 2 following complications related to cardiothoracic surgery to repair an atrioventricular septal defect and coarctation of the aorta and perform pulmonary artery banding. Chromosomal fragility studies using mitomycin C demonstrated increased breakage in the patient cells. BMF panel testing of peripheral blood demonstrated a homozygous missense RAD51C variant [NM_058216.2:c.773G>A; p.(Arg258His)]. This variant has previously been observed in the homozygous state in a single family with infants displaying a Fanconi anaemia-like disorder characterised by congenital heart disease, thumb, renal, intestinal and anal abnormalities. Experimental evidence is consistent with this variant being a hypomorphic mutant that results in increased chromosomal disruption and cell cycle arrest\textsuperscript{11-13}. The patient’s sister was subsequently referred for predictive testing with regard to her possible risk of breast and ovarian cancer.

Patient ID #47: A 64-year-old male presented with pancytopenia. He had been diagnosed in early adulthood with cerebellar ataxia of unclear cause. His brother and mother had also experienced cerebellar ataxia. Bone marrow aspirate and trephine demonstrated marked hypocellularity with some fibrosis and he commenced immunosuppressive therapy for presumed aAA. There was no response to immunosuppressive therapy and he subsequently commenced eltrombopag. Repeat bone marrow aspirate and trephine demonstrated a mild increased in blasts and cytogenetic clonal progression (46,XY,t(3;21)(q26;q22)) and eltrombopag was ceased. Targeted sequencing performed on peripheral blood demonstrated a CBL variant [NM_005188.3:c.1259G>C; p.(Arg420Pro)] at a variant allele frequency of 7.5\% consistent with the presence of clonal haematopoiesis. WES on peripheral blood identified a heterozygous missense variant in SAMD9L [NM_152703.2:c.2956C>T; p.(Arg986Cys)]. This variant has been reported in a family with ataxia-pancytopenia (ATXPC) syndrome and \textit{in vitro} evidence demonstrates that this variant is gain-of-function, enhancing the growth suppressing activity of SAMD9L\textsuperscript{14}. The patient subsequently presented with circulating blasts and bone marrow aspirate and trephine demonstrated progression to acute myeloid leukaemia. He died of infectious complications during induction chemotherapy. The patient’s children and family were subsequently referred for predictive testing.
**Patient ID #52:** A previously well 41-year-old female presented with severe anaemia. Bone marrow aspirate and trephine was performed and demonstrated marked erythroid hypoplasia. A provisional diagnosis of pure red cell aplasia was made based on bone marrow assessment. There was no evidence of parvovirus or thymoma and she was commenced on cyclosporine and corticosteroid. BMF panel testing on peripheral blood identified a large deletion of chromosome 5 spanning the region 5q14.3-33.2 which was confirmed with the finding of isolated deletion of 5q by conventional cytogenetics and a diagnosis of myelodysplastic syndrome with isolated del(5q) according to WHO 2016 diagnostic criteria was made (Supp Fig 6). On review, the marrow demonstrated subtle dysplastic features, in particular hypolobated megakaryocytes. The patient was commenced on lenalidomide with resolution of anaemia.

![Supplementary Figure 6. Interstitial deletion of the long arm of chromosome 5 spanning the region 5q14.3-33.2.](image)

**Patient ID #53:** An 11-year-old male was referred for genetic testing after developing persistent and severe pancytopenia following consolidation therapy containing cyclophosphamide (COG AALL1231) for T-acute lymphoblastic lymphoma. BMF panel testing identified two nonsense variants in FANCM [NM_020937.2:c.1972C>T; p.(Arg658*) and NM_020937.2:c.5101C>T; p.(Gln1701*)], both of which have been previously reported in cases of severe chemotherapy toxicity\(^{15, 16}\). There was no family history of a IBMFS or cancer predisposition, and no congenital malformations or previous abnormal blood parameters suggestive of Fanconi anaemia. Chromosome breakage studies using diepoxybutane on peripheral blood and tissue fibroblast cultures demonstrated a slight increase in the number of chromosome breaks compared to the control but below the number typically seen in Fanconi anaemia. Further chemotherapy was subsequently modified with reduced toxicity and ongoing response to therapy.
**Patient ID #60:** A 12-month-old male had presented with petechiae due to severe thrombocytopenia (platelets $5 \times 10^9/L$) at 8 months of age. He had microcephaly, orbital hypertelorism, leukoplakia and had so far demonstrated normal development. Investigations showed severe retinopathy and cerebellar hypoplasia. Telomere lengths of the patient’s mononuclear cells were low normal ($\geq 1^{st}$ and $<10^{th}$ percentile). BMF panel testing on peripheral blood detected two heterozygous missense variants in $TERT$ [NM_198253.2:c.3148A>G; p.(Lys1050Glu) and c.1670T>C; p.(Leu557Pro)]. Trans inheritance was inferred by the demonstration of the Lys1050Glu maternally and the Leu557Pro paternally. The Lys1050Glu has previously been reported in a kindred with familial idiopathic pulmonary fibrosis where the index case was shown to have short telomeres. In addition, in vitro telomerase activity assays have demonstrated some effect of the Lys1050Glu variant on enzyme function and processivity relative to wild type $TERT$ with structural analyses suggestive of a possible role for nucleic acid binding at or near this residue in the hThumb domain. The Leu557Pro is not present in population databases (gnomAD) and is undescribed in both the literature and relevant disease databases (ClinVar, Arizona State University Telomerase Database) but occurs in the highly conserved RNA binding (TRBD) domain responsible for $TERT$-$TER$ complex formation enabling telomeric DNA repeat synthesis. Both of these variants were shown to impair TERT function in in vitro assays (manuscript in preparation).

**Patient ID #62:** A 2-year-old male was born pre-term (33 weeks gestation). He required transfusion support for thrombocytopenia as a neonate. He experienced poor growth and multiple respiratory infections from the age of 4 months requiring hospitalisation. Bone marrow biopsy demonstrated marked left-shifted granulopoiesis with mild to moderate dysplasia of the megakaryocyte and erythroid lineages. Monosomy 7 was detected by metaphase cytogenetics. Chromosome breakage studies and faecal elastase were normal, and he had a normal appearing pancreas and skeletal survey. WES detected a $SAMD9$ variant [NM_017654.3:c.2318T>C; p.(Ile773Thr)] at a variant allele frequency of approximately 26%, consistent with the presence of a monosomy 7 as demonstrated by the BMF panel. The Ile773Thr was detected in a hair follicle sample and testing of parental samples demonstrated it to be de novo. The patient underwent a matched sibling donor allogeneic bone marrow transplant.

**Patient ID #67:** A 2-year-old female presented with severe macrocytic anaemia and watershed cerebral infarction. She had a history of mild hydrocephalus, subclinical hypothyroidism and ear anomalies. There was no significant family history. Bone marrow biopsy was hypocellular with...
markedly reduced erythropoiesis. Red cell adenosine deaminase (ADA) was elevated. An RPS19 missense variant [NM_001022.3:c.184C>T; p.(Arg62Trp)] was detected which was confirmed to be de novo through parental testing.

**Patient ID #69:** A 3-month-old male born to consanguineous parents was noted to have neutropenia after presenting at birth with respiratory distress and hypoglycaemia. There was no history of recurrent infections. He was noted to have an increased head circumference and subsequently underwent an MRI brain demonstrating a small subdural haemorrhage but no structural abnormalities. Examination revealed mild transaminitis and hepatosplenomegaly but no pancreatic insufficiency or other dysmorphic features. The parents reported a history of multiple early deaths in the family leading to the suspicion of an inherited disorder. Bone marrow aspirate and trephine was normocellular with non-diagnostic features. WES of a bone marrow sample detected a large deletion (1426 bp) in DNAJC21 involving part of exon 7 and the splice donor site at the exon 7/intron 7 boundary (c.972_983+1414del), which was confirmed in peripheral blood (Supp Fig 7A). The presence of wildtype reads suggested that this variant was heterozygous, and DNAJC21 was not with a region of homozygosity based on microarray analysis. RNA studies (by RNAseq) were consistent with the presence of a transcript with skipping of exon 7 (Supp Fig 7B). No other sequence variants or exon level deletions were detected. The family was referred for genetic counselling and segregation analysis.
Supplementary Figure 7. (A) WES demonstrating a c.972_983+1414del involving exon 7 of DNAJC21, with corresponding paired reads mapped to a region within intron 7. (B) Alignment of RNAseq reads demonstrating an aberrant transcript with skipping of exon 7.

Patient ID #76: A 33-year-old male presented in childhood with fingernail changes and moderate thrombocytopenia. Bone marrow biopsy demonstrated marked hypocellularity and a clinical diagnosis of dyskeratosis congenita was made. There was no family history of an IBMFS. BMF panel testing of peripheral blood detected a heterozygous missense TINF2 variant [NM_001099274.1:c.844C>T; p.(Arg282Cys)], which has previously been observed in multiple families as both de novo and as an inherited variant segregating with dyskeratosis congenita with an autosomal dominant mode of inheritance\textsuperscript{20, 21}. The TINF2 variant was subsequently detected in this patient’s son, who had severe thrombocytopenia (platelets 10-20 x 10\(^9\)/L) and bilateral exudative retinopathy (Revesz syndrome).

Patient ID #81: A 13-year-old male was being managed for severe refractory immune thrombocytopenia, which had been diagnosed when he presented aged 10 with platelets <10 x 10\(^9\)/L and marked epistaxis. Bone marrow aspirate and trephine demonstrated a moderately hypocellular marrow with significant dysmegakaryopoiesis. He was also found to be hypogammaglobulinaemic, with an IgG of 4.4g/L. Initially he had a moderate response to immunosuppression however this was not sustained. His family history was significant for his maternal grandfather having been diagnosed in adulthood with immune thrombocytopenia and his mother’s first cousin having had a splenectomy for refractory immune thrombocytopenia. WES of peripheral blood detected a heterozygous splice site variant in DDX41 [NM_016222.2:c.435-2_435-1delinsCA; p.?]. This variant occurs at the splice acceptor site of the intron 5/exon 6 boundary and is predicted to result in abnormal splicing. This
variant has been previously reported in one family with a history of myelodysplastic syndrome and leukaemia\textsuperscript{22}.

**Patient ID #82:** A 69-year-old female with a long standing history of neutropenia (approximately 15 years) had previously received chemotherapy for breast cancer diagnosed at age 66. The patient had no relevant family history. A bone marrow biopsy performed for recent progressive cytopenias demonstrated moderate to marked hypocellularity with trilineage dysplasia and 6% blasts. Conventional cytogenetics did not identify any abnormalities. A \textit{DDX41} missense variant [\textit{NM}_016222.2:c.517G>A; \textit{p.(Gly173Arg)}] was detected which has recently been described in five patients presenting with a similar clinical phenotype\textsuperscript{23}. Consistent with the five cases reported to date, deep sequencing of \textit{DDX41} in this patient also identified a recurrent somatic \textit{DDX41} variant [\textit{NM}_016222.2:c.1574G>A; \textit{p.(Arg525His)}] present at 3.3% variant allele frequency (Supp Fig 8).

Supplementary Figure 8. Somatic \textit{DDX41} Arg525His detected in a 69-year-old female with a germline Gly173Arg \textit{DDX41} variaint.

**Patient ID #85:** A 33-year-old female was diagnosed with Fanconi anaemia in childhood as a result of short stature, short thumbs, bilaterally absent index fingers and mild cognitive and hearing impairment. She subsequently experienced premature ovarian failure, primary biliary cirrhosis and severe chronic obstructive pulmonary disease despite no personal smoking history. BMF panel testing demonstrated two heterozygous \textit{FANCI} variants [\textit{NM}_001113378.1:c.3184C>T; \textit{p.(Gln1062*)} and \textit{NM}_001113378.1:c.3041G>A; \textit{p.(Cys1014Tyr)}]. The Cys1014Tyr missense variant has been described
in three patients with a clinical diagnosis of Fanconi anaemia, one of whom was homozygous for the Cys1014Tyr\textsuperscript{24} and two in the compound heterozygous state with truncating FANCI variants\textsuperscript{25, 26}.

**Patient ID #87:** A 49-year-old male presented with pancytopenia. A bone marrow biopsy demonstrated marked hypocellularity. No PNH clone was detected on peripheral blood. A diagnosis of aAA was made and the patient commenced immunosuppressive therapy with anti-thymocyte globulin/cyclosporine with minimal improvements in blood counts. BMF panel testing of peripheral blood detected a novel TERT variant [NM_198253.2:c.1223T>C; p.(Leu408Pro)]. In addition, a frameshift BCOR variant [NM_017745.5:c.3549_3561dup; p.(Val1188Metfs*27)] was also detected (16% variant allele frequency). The finding of a TERT variant prompted telomere length studies which were demonstrated to be <1\textsuperscript{st} percentile (flow FISH). Additional testing by ddPCR identified a TERT promoter variant [NM_198253.2:c.-124C>T], which has been described in patients with a clinical diagnosis of a telomeropathy and germline TERT/TERC variants\textsuperscript{27}. There were no phenotypic features of dyskeratosis congenita in the patient. He subsequently underwent an allogeneic stem cell transplant from a sibling with documented normal telomere length by flow FISH.

**Patient ID #96:** A 34-year-old male was referred for pancytopenia noted during work up of liver lesions which were subsequently demonstrated to be hepatocellular carcinoma. He had a past history of mandibular squamous cell carcinoma at age 32 for which he had undergone surgery and radiotherapy. He had experienced significant radiation toxicity with residual scarring resulting in the need for ongoing feeding via percutaneous endoscopic gastrostomy tube. His family history was notable for his sister having been diagnosed with myelodysplastic syndrome in her 20s. BMF panel testing demonstrated two heterozygous FANCA missense variants [NM_000135.2:c.2852G>A; p.(Arg951Gln) and NM_000135.2:c.3971C>T; p.(Pro1324Leu)] both of which have been reported in multiple individuals with Fanconi anaemia in the compound heterozygous state\textsuperscript{28-32}. Peripheral blood chromosomal fragility studies using diepoxybutane demonstrated increased breakage. Hepatocellular carcinoma therapy was then tailored to avoid undue toxicity.

**Patient ID #104:** A 20-year-old female had been diagnosed with severe congenital neutropenia in infancy after presenting with culture negative febrile neutropenia. In addition she had significant cognitive impairment of unclear cause. She received life-long G-CSF therapy and experienced intermittent infections (predominantly skin). Her 15-year-old brother also experienced severe
congenital neutropenia requiring life-long G-CSF, significant cognitive impairment and hypertension. Her father had previously been diagnosed with cyclical neutropenia and also had some mild cognitive impairment. WES detected a heterozygous in-frame deletion in SRP54 [NM_003136.3:c.349_351del; p.(Thr117del)]. The Thr117del is the most commonly described variant in SRP54 in case series of severe congenital neutropenia to date and has been described as both a de novo and an inherited variant segregating with an autosomal dominant mode of inheritance. The SRP54 Thr117del was subsequently detected in the patient’s brother and father.

**Patient ID #106**: A 4-year-old female presented with a fluctuating mild anaemia since birth. Bone marrow biopsy was moderately hypocellular with mild dyserythropoiesis. An elevated red cell ADA was demonstrated. Her mother had been diagnosed with a transfusion-dependent macrocytic anaemia of unclear cause at age 13 and continued to receive regular red blood cell transfusions into adulthood. BMF panel testing on peripheral blood of the child demonstrated a heterozygous splice site variant in RPS7 [NM_001011.3:c.75+1G>T; p.?] . The c.75+1G>T occurs at the canonical splice donor site on the exon 2/intron 2 boundary and is predicted to result in abnormal splicing by in silico predictors. This variant has not been reported in population databases (gnomAD), ClinVar, or the literature, however other RPS7 splice site variants have been reported in patients with Diamond-Blackfan anaemia. This variant was subsequently also detected in the peripheral blood of the child’s mother.

**Patient ID #107**: An 18-year-old female had been diagnosed with severe congenital neutropenia shortly after birth. She had received life-long G-CSF therapy and suffered from intermittent skin and respiratory tract infections. She had been considered for allogeneic stem cell transplant, however neither a related nor unrelated donor could be identified. BMF panel testing demonstrated a heterozygous nonsense variant in ELANE [NM_001972.2:c.684C>A; p.(Tyr228*)]. In addition, a low level acquired variant was detected in CSF3R [NM_156039.3:c.2308C>T; p.(Gln770*)] at a variant allele frequency of 2.9%. The ELANE Tyr228* has previously been reported in patients with severe congenital neutropenia (including in association with myelodysplastic syndrome/acute myeloid leukaemia) and cyclical neutropenia.

**Patient ID #118**: A 21-year-old female was referred with fluctuating mild-moderate cytopenias and infections since the age of 15. Her family history was significant for a paternal aunt and uncle both...
having died from acute myeloid leukaemia in young adulthood. Peripheral blood flow cytometry of lymphocyte subsets demonstrated B and NK cell cytopenias. Bone marrow aspirate and trephine was moderately hypocellular with erythroid hyperplasia, dyserythropoiesis and dysmegakaryopoiesis. Monosomy 7 was detectable by conventional cytogenetics. Targeted sequencing performed on bone marrow aspirate demonstrated an acquired pathogenic SETBP1 variant [NM_015559.2:c.2602G>A; p.(Asp868Asn)] typically observed in myeloproliferative/myelodysplastic overlap entities but having also been seen in aAA41. BMF panel testing of peripheral blood demonstrated a heterozygous synonymous variant in GATA2 [NM_032638.4:c.351C>G; p.(Thr117=)]. RNA studies have previously demonstrated that this variant results in the introduction of a cryptic splice donor site, leading to an aberrantly spliced transcript with a 136 bp internal deletion resulting in a frameshift and a truncated GATA2 protein. This variant has previously been reported in patients with clinical features of a GATA2 haploinsufficiency syndrome, including in a mother and her three children who were all affected with GATA2 haploinsufficiency syndrome-associated clinical features, and in an unrelated individual with immunodeficiency with no known family history. The patient underwent a matched unrelated donor allogeneic stem cell transplant and the family has been referred for predictive testing.

**Supplementary References**


