results: (i) Double-positive, (ii) Double-negative, (iii) Equivocal. Group (iii) included any case that did not belong to groups (i) and (ii), ie those with equivocal results on one or both tests and those with a positive result on one test and negative result on the other. Groups (i) and (ii), collectively referred to as "concordant," were used to determine the optimal Ct cutoff that maximized the agreement between tissue PCR results and double positivity/negativity.

Biopsies were obtained from colon, duodenum, stomach, esophagus, and lung in 59 (39%), 44 (29%), 37 (24%), 7 (5%), and 4 (3%) cases, respectively. One hundred twenty-two (80%) cases were concordant (105 double-negative and 17 double-positive) and the remainder were equivocal. Viremic cases were marginally significantly more likely than non-viremic cases to be equivocal (28% vs. 14%, respectively; $P = 0.055$). Pathological evidence of GVHD was present in 57% vs. 52% of concordant vs. equivocal cases, respectively ($P = 0.68$). Similarly, these groups were not different with regards to the presence of clinical evidence of GVHD (82% vs. 90%, respectively). The optimal PCR Ct value for classification of concordant cases was 40, with good overall performance (AUC 0.91, $P < 0.001$), sensitivity 94%, specificity 79%, positive predictive value (PPV) 42%, and negative predictive value (NPV) 99%. Using this cutoff, 45% of equivocal cases were classified as negative. Table 1 shows the distribution of patients across H&E/IHC and PCR subgroups. Among viremic H&E/IHC-concordant cases, tissue PCR had a sensitivity of 100%, specificity of 50%, PPV of 44%, and NPV of 100%. Among non-viremic H&E/IHC-concordant cases, these numbers were 80%, 91%, 36%, and 99%, respectively. In this analysis on viremic and non-viremic cases, 31% and 62% of equivocal cases were classified as negative, respectively.

One of the challenges in the diagnosis of CMV disease is H&E/IHC-equivocal cases. Avoiding potentially toxic anti-CMV treatment can be beneficial especially in non-viremic patients with negative tissue PCR. We demonstrate that a negative tissue PCR can be used to rule out CMV disease in H&E/IHC-equivocal cases. Although minimal spatial heterogeneity in the CMV target organ is assumed, we cannot rule out this possibility. Furthermore, we performed tissue PCR on FFPE specimens. The results may change if PCR were on fresh tissue, as it would be in the real-life setting. We expect the sensitivity of fresh tissue PCR to increase and specificity to decline. Until replicated in a controlled prospective study using fresh tissue samples, our results should not be used to guide treatment decisions.

Our goal in this innovative, but preliminary, study was to assess the value of tissue PCR as an adjunct to H&E/IHC in the diagnosis of CMV disease, especially in equivocal cases. Future research could focus on clinical outcomes of non-viremic equivocal cases and whether anti-CMV treatment can be safely withheld in this group of patients. We could not address this question in the present work because all of our equivocal cases received anti-CMV treatment. Given the possibility of sampling error when using PCR on tissue biopsies, applying the same technique to fluid samples such as bronchioalveolar lavage or stool samples may be of value in future research.

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Author Contributions

AR, JFD, and GAS designed the study. KRV performed H&E and IHC. RSB and KMW performed PCR. AR wrote the manuscript. All authors critically reviewed the manuscript and approved the final draft

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Gray platelet syndrome: Novel mutations of the NBEAL2 gene

Gray platelet syndrome (GPS) is a rare inherited macrothrombocytopenia characterized by reduction of α-granules in platelets and megakaryocytes associated with mild-to-moderate bleeding and myelofibrosis [1]. As reported in at least 28 unrelated families [1], GPS is caused by mutations of *NBEAL2*, the gene encoding for the neurobeachin-like-2 protein. NBEAL2 is a member of the family containing the BEACH (BEige And Chediak Higashi) domain, a conserved region involved in vesicular trafficking that may be critical for the α-granule development [2]. Here, we report novel mutations of *NBEAL2* in two affected individuals (P1 and P2), who were previously diagnosed with immune thrombocytopenia (ITP) and then suspected to have GPS because of absence of azurophilic granules on May-Grünwald-Giemsa staining. In P1, sequencing analysis identified an homozygous missense variant (c.6212G > C; p.Arg2071Pro; Supporting Information Fig. S1). Since her parents were not available for the segregation analysis, we hypothesised that the two mutant alleles were identical by descent because of homozygosity of all the polymorphic markers at the *NBEAL2* locus (data not shown). Moreover, the potential hemizygous condition was excluded using statistical analyses of *NBEAL2* amplicon coverage as previously reported (Supporting Information Fig. S1C) [3]. In P2, we detected one maternal nonsense (c.3839C > T; p.Arg1280*) and one paternal missense (c.6477C > G; p.His2159Gln; Supporting Information Fig. S1). The three *NBEAL2* variants are reported in SNPs databases but with a minor allele frequency <0.01%.

Whereas the deleterious effect of nonsense mutations is usually associated with loss of function, the pathogenic role of the missense variants is not readily obvious. Several bioinformatics tools have been developed to predict the effect of amino acid substitutions. Among these, the CADD (Combined Annotation Dependent Depletion) score is relatively high for both p.Arg2071Pro and p.His2159Gln (Table 1). Moreover, the two affected residues are well conserved in orthologs of different species, suggesting that they are important for the NBEAL2 activity (Supporting Information Fig. S1G). Indeed, they affect the BEACH domain, a region that is highly homologous with that of another two human proteins, neurobeachin (NBEA) and LPS-responsive vesicle trafficking, beach and anchor protein (LRBA; Supporting Information Fig. S2A) [2]. Even in NBEA and LRBA residues Arg2071 and His2159 are conserved (Supporting Information Fig. S2B). Using the crystal structure of the BEACH domain in NBEA and LRBA, we found that Arg2071 is placed in a rigid turn of a helical hairpin and is involved in a hydrogen bond with Glu2270 (Supporting Information Fig. S2C). His2159 is unusually shielded in the core of the structure and not exposed to the solvent because of a polar interaction with Thr2333. Of note, residues Glu2270 and Thr2333 are also conserved in NBEAL2, NBEA, and LRBA (Supporting Information Fig. S2B). Therefore, we can hypothesize that the Arg2071Pro and His2159Gln amino acid substitutions affect the energetics of the hydrogen bonding, which needs to be optimal to allow rapid sampling and kinetics of folding, conferring stability to the structure. Although the specific function of the BEACH domain remains unclear, it is likely to interact with the PH domain and form a large groove as a binding site for ligands. Therefore, p.Arg2071Pro and His2159Gln, as well as the others missense mutations affecting the BEACH domain, are likely to prevent interactors from binding NBEAL2 (Supporting Information Fig. S2A).

Consistent with the identification of mutations in the *NBEAL2* gene, the two affected individuals had thrombocytopenia with platelet anisocytosis and in their platelets α-granules were almost absent (Table 1; Supporting Information Fig. S3). Accordingly with our previous study [4], the parents of P2 had a moderate reduction of the α-granule number. Although we cannot exclude genetic heterogeneity in GPS, *NBEAL2* is the only gene known whose biallelic mutations are associated with severe α-granule deficiency.

Considering the expression level of glycoproteins (GP) on the platelet membrane, GPI/IX/V and GPIIb/IIIa were expressed at the normal levels whereas GPVI was slightly reduced at least in P1 (Table 1). Indeed, after stimulation with convulxin, a direct agonist of collagen receptor GPVI, the platelets of P1 were defective in P-selectin exposure, dense granule secretion, and GPIIb/IIIa activation. Considering that defects of platelet aggregation with collagen have previously been reported in GPS [1], a systematic evaluation of

TABLE 1. Molecular and clinical features in GPS patients[a]

Patient	**P1** (25-year-old female)	**P2 (**10-year-old male)
NBEAL2 mutation	c.6212G>C (exon 38) p.Arg2071Pro (CADD 20.1)†	c.3839C>T (exon 27) p.Arg1280* c.6477C>G (exon 40) p.His2159Gln (CADD 19.8)†
Platelet count indicated as $\times 10^9$/L	27–66 (cell counter)	36 (cell counter) 54 (microscopy)
Mean platelet volume or mean platelet diameter	12.1–13.5 fL	3.96 μm (2.27–3.08 μm, normal range)
α-granule content	Almost absent (electron microscopy)	Almost absent (TPS-1 immunofluorescence assay)
Platelet aggregation after stimulation with ADP, epinephrine, and arachidonic acid	nd	Normal
Expression level of		
GPIIb/IIIa (CD41/CD61)	Normal	Normal
GPVI	Slightly decreased	nd
Thrombin receptors	nd	Reduced (PAR1)
P-selectin exposure after stimulation with		
ADP	Reduced	Reduced
Thrombin or TRAP-6	Reduced (Thrombin)	Reduced (TRAP-6)
Convulxin	Reduced	nd
Dense granule secretion after stimulation with		
Thrombin	Reduced	nd
Convulxin	Reduced	nd
GPIIb/IIIa activation recognized by PAC-1 after stimulation with		
ADP	Normal	Normal
Thrombin or TRAP-6	Reduced (Thrombin)	Reduced (TRAP-6)
Convulxin	Reduced	nd
Other associated finding		
Diagnosis of ITP	Yes	Yes (refractory to corticosteroids
Bleeding diathesis	Absent	Severe epistasis requiring transfusions
Spleen size	Normal	Enlarged
Myelofibrosis	Slight to moderate	No
Emperipolesis	nd	Yes
Vitamin B12	nd	Increased
Other features	Leukopenia, due to lymphocytopenia (0.8–1.4 $\times 10^9$/L)	No

[a] Materials and methods for data indicated in the Table are reported in Supporting Information.
[b] CADD, Combined Annotation Dependent Depletion score.
Abbreviation: nd, not determined.

the GPVI expression would be of interest to ascertain whether its reduction is a symptomatic feature in GPS. Similarly, the PAR1 mediated platelet response could be another common defect in the disease, as a recent study showed low expression level of the PAR1 and PAR4 thrombin receptors [5]. Therefore, PAR1 was analysed in P2, confirming the data from the literature. As a consequence, after stimulation with thrombin or thrombin receptor activating peptides (TRAP-6), we observed the same defective platelet activation as that observed with convulxin (Table 1).

Consistent with the identification of the *NBEAL2* mutations, P1 had slight/moderate bone marrow fibrosis but no enlarged spleen [6]. Megacaryocytes were small in size with hypolobulated nuclei and extensive emperipolesis. Of note, the patient had 2.7–6.0 $\times 10^9$/L white blood cells with underlying lymphocytopenia of 0.8–1.4 $\times 10^9$/L but without a history of recurrent or severe infections (Table 1). Being not reported in GPS, this mild lymphocytopenia could be independent of α-granules deficiency.

On the contrary, P2 had increased serum B12, splenomegaly, and emperipolesis but not myelofibrosis (Table 1). Considering that P1 is older than P2, there is significant variable expressivity of the disease between the two patients. This is also consistent with the degree of their bleeding diathesis. Whereas P1 had negative bleeding history and underwent two elective uneventful caesarean sections with prophylactic treatments, P2 suffered from severe epistasis that required platelet transfusions. Although the α-granule deficiency is of similar extent in the two patients, we cannot exclude that the homozygous p.Arg2071Pro mutation identified in P1 allows NBEAL2 to retain some residual activity.

In conclusion, we report novel biallelic *NBEAL2* mutations associated with variable expressivity of GPS. Moreover, we support the hypothesis that defective expression of GPVI and the thrombin receptors on the GPS platelets are additional signs of the disease, whose recognition is fundamental to prevent misdiagnosis of ITP and undue therapy.

AUTHOR CONTRIBUTIONS

All authors agreed with the content of the manuscript and approved the final version.

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