Congenital amegakaryocytic thrombocytopenia: clinical and biological consequences of five novel mutations

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ABSTRACT

Background and Objectives

Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare, autosomal recessive disorder induced by mutations of the gene coding for thrombopoietin (TPO) receptor (c-MPL). Patients initially present with isolated thrombocytopenia that subsequently progresses into pancytopenia. Although the mechanisms leading to aplasia are unknown, the age of onset has been reported to depend on the severity of the c-MPL functional defect. To improve our knowledge in this field, we studied clinical and biological features of five new patients.

Design and Methods

We diagnosed five CAMT patients, identified *c-MPL* mutations, including five novel alterations and investigated relationships between mutations and their clinical-biological consequences.

Results

In all cases, platelet c-MPL and bone marrow colonies were reduced, while serum TPO levels were elevated. We also documented that the percentage of bone marrow cells expressing tumor necrosis factor-α and interferon-γ was increased during pancytopenia as compared to controls, suggesting that, as in other bone marrow failure diseases, these inhibitory cytokines contributed to the pancytopenia. Contrary to previously published data, we found no evidence of correlations between different types of mutations and the clinical course.

Interpretation and Conclusions

These results suggest that therapies, such as hematopoietic stem cell transplantation, which are potentially curative although associated with a risk of treatment-related mortality, should not be postponed even in those CAMT patients whose *c-MPL* mutations might predict residual activity of the TPO receptor.

Key words: congenital amegakaryocytic thrombocytopenia, CAMT, *c-MPL*, thrombopoietin receptor, mutations.

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Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare, autosomal recessive bone marrow failure syndrome, characterized by early onset of isolated hypomegakaryocytic thrombocytopenia that later evolves into tri-lineage marrow aplasia. The disease is caused by a defective thrombopoietin (TPO) receptor (c-MPL).[1] The thrombopoietin receptor is defective due to mutations in the *c-MPL* gene.[1] TPO binding stimulates both early and late phases of megakaryocytopoiesis, increasing the number, size, and ploidy of megakaryocytes, and promoting the expression of platelet-specific markers. However, TPO has no or little effect on platelet shedding from megakaryocytes.[2] TPO is not only the most important growth factor for megakaryocytopoiesis but it is also involved in maintaining the numbers of hematopoietic stem cells, this fact contributing to explain the occurrence of both thrombocytopenia and pancytopenia in CAMT patients. Consistent with this hypothesis, animals deficient for TPO or c-Mpl have a 90% reduction of megakaryocytic precursor cells and a 60-80% decrease of both erythroid and myeloid progenitors.[3] At least 28 different *c-MPL* mutated alleles have been so far identified from 32 unrelated CAMT families.[1,4-11] Beside these mutations associated with CAMT, a gain-of-function mutation of c-MPL is responsible for familial essential thrombocythemia.[12] Interestingly, different types of mutations have been associated with different phenotypes, allowing patients to be subdivided into two groups. Mutations predicted to result in a complete loss of function of the TPO receptor led to more severe thrombocytopenia and early onset of pancytopenia, whereas missense mutations were associated with transient increases of platelet counts during the first year of life and late or no development of pancytopenia.[9] We recently diagnosed CAMT in five unrelated Italian patients and identified five novel mutations of *c-MPL*. At variance with previous reports, in our group of patients neither the type of mutations nor the other investigated functional or molecular parameters predicted the severity of the disease.

Design and Methods

Patients

The patients, all from Italian unrelated, non-consanguineous families, were referred to the IRCCS San Matteo Hospital Foundation (Pavia) and the Giannina Gaslini Children's Hospital (Genoa) because of congenital thrombocytopenia of unknown origin. In all of them thrombocytopenia was severe, platelet counts never exceeding 30×10^9/L. Petechiae were present since birth or were noted during the first months of life in all patients, with the only exception of patient CAMT2, whose petechiae became manifest at the age of 20 months. Patient CAMT4 had an intraparenchymal brain hemorrhage 3 days after birth. Patient CAMT5 died at the age of 51 months from brain hemorrhage. The diagnosis of CAMT was made based on the presence of a non-syndromic thrombocytopenia with normal mean platelet volume, severely reduced numbers of bone marrow megakaryocytes and identification of *c-MPL* mutations. The ages at diagnosis and the time courses of blood counts are reported in Table 1. Before diagnosis, patient CAMT1 had received high dose intravenous immunoglobulins (IVIg), while the other four patients had been treated with steroids plus IVIg without any improvement of the thrombocytopenia. All patients developed pancytopenia as a result of severe, general bone marrow aplasia, documented by a marrow cellularity of less than 20%, at an age comprised between 22 and 49 months. Interestingly, patient CAMT4 become aplastic and red cell transfusion-dependent at the age of 49 months, but 8 months later his hemoglobin values spontaneously improved and he required no further transfusions, while neutropenia and thrombocytopenia persisted over time. Patients CAMT1 and CAMT2 were successfully transplanted when they were 40 and 50 months old with bone marrow cells after having received a fully myeloablative conditioning regimen including a combination of busulfan, fludarabine and thiotepa. Now, 14 and 10 months after transplant, they have normal blood counts. CAMT 4 was transplanted when he was 78 months old with one antigen mismatched (locus A) unrelated cord blood after a reduced intensity conditioning regimen containing fludarabine and thiotepa. The patient showed full donor chimerism resulting in complete hematopoietic reconstitution. After having suffered from acute graft-versus-host diease (GVHD) grade IV, now, 6 months after the graft, he has extensive chronic GVHD. All the patients' parents and the carrier sister of patient CAMT1 had normal blood counts without a bleeding tendency. The institutional review board of Fondazione IRCCS Policlinico San Matteo, University of Pavia, Italy, approved the study. Blood and marrow samples from patients, as well as from healthy donors, were collected after obtaining written informed consent.

Screening for mutations of the c-MPL gene

DNA was extracted from peripheral blood leukocytes using the Puragene DNA isolation kit (Gentra Systems, Minneapolis, MN, USA). The coding regions of *c-MPL* were analyzed using oligonucleotides that we designed in introns for the amplification of exons and their flanking sequences (oligonucleotide sequences available upon request). Polymerase chain reactions (PCR) were carried out in a total volume of 50 µL with 25 ng of genomic DNA, 20 pmoles of each primer, 200 µM dNTPs, $MgCl_2$ 1.5 mM, 1,25 U of AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA, USA) and 5 µL of the corresponding 10× PCR buffer. After an initial denaturation step at 94°C for 12 minutes, amplification was performed for 30 cycles (denaturation at 94°C for 30 seconds, annealing at the specific temperature for single pairs of primers for 40 seconds, and extension at 72°C for 40 seconds). Direct sequencing was performed using dye terminator chemistry following the instructions in the user's manual (Big Dye Terminator Cycle Sequencing kit, Applied Biosystems). Electrophoresis of the cycle-sequencing products was car-

Table 1. Patients' characteristics and results of blood counts and bone-marrow colony-forming unit assays.

Patient/Age at diagnosis (months)/Clinical findings	*Age (months)*	*Platelets ($\times10^9$/L)*	*WBC ($\times10^9$/L)*	*Neut ($\times10^9$/L)*	*Hb (g/dL)*	*BFU-E**	*CFU-GM**	*CFU-Mk#*
CAMT1/32/Petechiae since	25	20	6.2	2	12.4	9	29	nd
birth	27	19	9.2	4.3	13	4	6	0
	38	8	4.3	0.6	8.7	nd	nd	nd
CAMT2/24/Petechiae since	30	15	12.1	2.6	11.8	4	9	0
the age of 20 months	40	9	8.6	1.9	10.7	5	11	2
	46	6	4.3	0.6	7	0	0	0
CAMT3/22/ Petechiae since	16	17	10.5	1.5	10.7	nd	nd	nd
birth	22	9	8.3	0.7	11.3	32	34	4
CAMT4/40/Petechiae since	49	3	5.7	0.6	8.1	nd	nd	nd
birth, brain hemorrhage 3	73	6	3.5	0.5	10.6	35	29	6
days after birth								
CAMT5/36/Petechiae since	29	13	4.2	0.9	8.8	nd	nd	nd
birth, fatal brain hemorrhage								
at the age of 51 months								

*Number of colonies per *2×10^4 or # 1×10^5 LDBMC; each number represents the mean number of colonies of an assay performed in duplicate. Normal values (ranges): BFU-E 35-55, CFU-GM 30-50, CFU-Mk 15-55. nd: not done.*

ried out with an ABI sequencing analysis software (from Applied Biosystems). To confirm and ascertain the segregation of the D128Y mutation within CAMT1 family, genomic DNA was amplified using primers 1F (5'-agcttcctgaagggaggatg-3') and 3R (5'-ggtctggtaataggggtatc-3') 1038 bp. Because the substitution creates a restriction enzyme site for R*sa*I, PCR products were purified, digested with the specific enzyme, and electrophoresed on 2% agarose gel (Figure 1).

Hematopoietic colony assay

Bone marrow aspirates were utilized for hematopoietic colony assays. Light density bone marrow cells (LDBMC) were obtained by centrifugation of a diluted bone marrow sample on a Ficoll (Amersham-Pharmacia Biotech, Uppsala, Sweden) gradient (1077 g/mL) at 400 g for 30 min. After washing, 2×10^4 LDBMC were plated in a standard methylcellulose assay in the presence of 30% fetal calf serum (Hyclone, Logan, UT, USA), erythropoietin (3 U/mL), interleukin-3, granulocyte-macrophage colony-stimulating factor (both at 20 ng/mL) and stem cell factor (50 ng/mL). All growth factors were purchased from Peprotech EC, London, UK. After 14 days at 37°C in 5% CO_2, colonies from duplicate cultures were scored as BFU-E and CFU-GM according to standard criteria.[13] Megakaryocyte colonies (CFU-Mk) were assessed by plating 1×10^5 LDBMC on double-chamber slides in a collagen-based medium supplemented with thrombopoietin (50 ng/mL), interleukin-3 (10 ng/mL) and interleukin-6 (10 ng/mL) (StemCells, Vancouver, BC, Canada). After 12 days of incubation at 37°C in 5% CO_2, the slides were dehydrated and immunostained to detect glycoprotein (GP) IIb-IIIa positive colonies. CFU-Mk colonies were scored according to previously reported criteria.[14,15] As a control, LDBMC from four healthy subjects donating marrow cells for allogeneic transplantation were cultured using the same method as that reported for the CAMT patients.

Flow cytometric analysis of membrane glycoproteins

Surface expression of platelet GPs was investigated in platelet-rich plasma by flow cytometry with an Epics XL flow cytometer (Coulter Corporation, Miami, FL, USA) as previously reported.[16] The following monoclonal antibodies from Immunotech (Marseille, France) were used: SZ21 that recognizes GPIIIa (β_3/CD61), P2 recognizing GPIIb (α_{IIb}) in the intact complex with GPIIIa (α_{IIb}/β_3 CD41), SZ2 against GPIbα (CD42b), SZ1 against GPIX (CD42a), Gi9 against GPIa (CD 49b), FA6-152 against GPIV (CD 36). We also used clone 167620 from R&D Systems (Minneapolis, MN, USA) against c-MPL and SW16 (CLB, Amsterdam, The Netherlands) against GPV (CD42d). MO2 (Coulter Corporation) was used as a negative control. Fluorescein isothiocyanate-conjugated goat anti-mouse IgG (GAM-FITC) was purchased from Coulter Corporation. A sample from a healthy donor was run with the patients' samples (cells from the same healthy donor were used for all patients). For c-MPL evaluation, we calculated the ratio between the mean fluorescence intensity (MFI) obtained with the antibody against c-MPL and the isotype control.[17] For all other antigens, MFI in patients was expressed as a percentage of the intensity in controls.

Serum TPO levels

Serum TPO was measured using the commercial enzyme-linked immunosorbent assay (ELISA) kit, Quantikine Human TPO Immunoassay, from R&D Systems.

Intracellular expression of tumor necrosis factor (TNF)-α and interferon (IFN)-γ

Bone marrow heparinized samples from patients and 10 healthy controls donating marrow cells for allografts were collected from the posterior iliac crest after having obtained written informed consent. Cells were isolated through centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala,

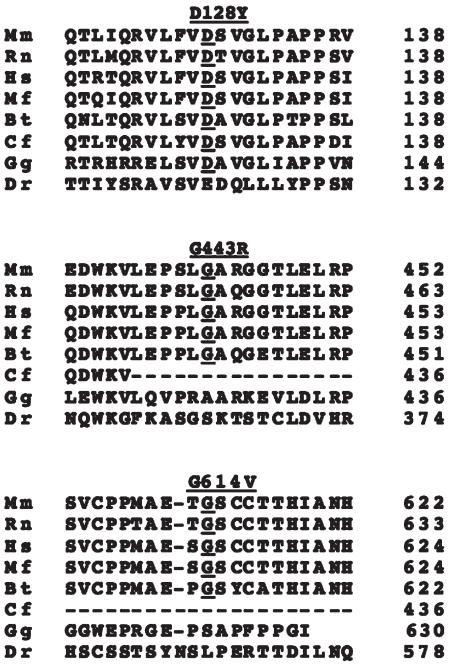

Figure 1. ClustalW alignment of the amino acid sequences of c-MPL from different species. The D, G, and G residues (underlined) at positions 128, 443, and 614, respectively, are conserved through evolution. Hs, *Homo sapiens* (NP-005364); Mm, *Mus musculus* (AAI03516); Rn, *Rattus norvegicus* (XP-345573); Cf, *Canis familiaris* (XP-853442; only 435 N-terminus amino acids of the TPO receptor are reported in database); Bt, *Bos taurus* (XP-888055); Gg, *Gallus gallus* (AAT45555); Dr, *Danio rerio* (AAQ82785).

Sweden) density gradients, washed in phosphate-buffered saline (PBS, Gibco, Carlsbad, CA, USA), frozen in 10% dimethylsulfoxide (DMSO), stored in liquid nitrogen and then thawed for use. After thawing, cells were processed as described elsewhere.[18] Briefly cells were cultured (1×10^6 cells/mL) for 5-6 hours at 37°C, 5% CO_2, in RPMI 1640 supplemented with 10% FCS, 100 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, and 2mmo/L penicillin/streptomycin in the presence of 50 ng/ml PMA, 250 ng/mL calcium ionophore and 5 mg/mL brefeldin A (Sigma, St Louis, MO, USA). After culture, cells were washed twice in PBS with 1% BSA and subsequently stained for 30 min at 4°C with FITC-conjugated monoclonal antibodies specific for the CD3 antigen. Cells were then fixed for 20 min at 4°C in 4% paraformaldehyde, washed and permeabilized in the presence of 0.1 % saponin. Finally, re-suspended cells were incubated for 30 min at 4°C in the presence of optimal concentrations of phycoerythrin (PE)-labeled anti-TNF-α and anti-IFN-γ antibodies. Negative staining controls were represented by PE or FITC-conjugated isotype-matched monoclonal antibodies of irrelevant specificity and by ligand blocking control in which the fluorochrome-conjugated antibodies had been preincubated with appropriate concentrations of recombinant cytokines according to the suppliers' instructions.

Flow cytometric analysis was performed using a FACScan cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA) and the gate was selected on the lymphocytes. In both patients and controls results were expressed as: (i) percentage of $CD3^+$ and $CD3^-$ marrow cells containing intracellular IFN-α or TNF-γ. For all subsets of cells (TNF-$\alpha^+/CD3^+$, TNF-$\alpha^+/CD3^-$, IFN-$\gamma^+/CD3^+$ and IFN-$\gamma^+/CD3^-$), single percentage values were derived from each patient, whereas mean percentage values ± SD were calculated in controls; (ii) mean ratio of relative fluorescence intensity (MRFI), which was calculated on the same cell fractions as follows: MFI of cytokine staining/MFI of irrelevant isotype-matched monoclonal antiobody staining. As above, for every subset of cells single MRFI values were derived from each patient, whereas mean values ± SD were calculated in controls. $CD3^-$ cells were intended as all cells not staining for CD3, including monocytes and hematopoietic progenitors. Further flow cytometric characterization was precluded by lack of cells.

Results

Identification of c-MPL mutations

Since hematologic investigations strongly suggested a diagnosis of CAMT, the 12 exons of *c-MPL* were screened for mutations in the probands of our families. We identified three missense, two nonsense and two frame-shift mutations (Table 2). In patient CAMT1, a novel c.382G>T mutation in exon 3, present in a homozygous state, led to a novel amino acid substitution at position 128 (D128Y). The mutation creates a site for the restriction enzyme R*sa*I, which was used to ascertain the segregation in both parents and heterozygosity in one sister (Figure 1).

In patient CAMT2 we identified another novel missense mutation, c.1327G>C (G443R) and a known nonsense substitution, c.127C>T (R43X).[5] Whereas G443R was transmitted from the father, the R43X mutation was not detected in the mother. However, highly informative microsatellite markers showed segregation within the family (*data not shown*) and repeated mutational analysis confirmed the presence of the R43X allele in the affected child and its absence in the mother, suggesting that either the mother is a mosaic for R43X or the mutation occurred as a *de novo* event in the patient. RNA from platelets was not available for determining whether the two mutations were in *cis* or in *trans*.

Patient CAMT3 carried two novel mutations, c.189C>G and c.1841G>T, leading to a nonsense (Y63X) and missense (G614V) substitution, respectively. Patients CAMT4 and CAMT5 were compound heterozygotes for the same two mutations transmitted from parents in both cases. Although they came from the same province in southern Italy, we were unable to identify genealogical relationships between these families. The first mutation was a dinucleotide deletion in exon 3 (c.235-236delCT; L79EfsX84) previously described,[5] resulting in a premature truncated c-MPL protein. The second allele carried a novel deletion of one of two adjacent G that define the boundary between exon and intron 11 (c.1653delG or c.1653+1delG). Since

the deletion of one G does not severely affect the predicted score for donor splice, which was 0.99 and 0.93 (as calculated at *http://www.fruitfly.org/seq_tools/splice.html*) for the wild type (CCGgtgagtgt) and mutated (CCCgtgagtgt), exon/intron junctions, respectively, the removal of intron 11 is expected to occur correctly during the RNA processing phases. If this was the case, the single nucleotide deletion would determine the translation of 76 novel residues before encountering a stop codon (K553RfsX75). Since patients' RNA samples were not available, we cannot exclude that the mutation might interfere with the splicing of intron 11, leading for instance to recognition of crypt donor splice sites or retention of intron 11. The three novel missense mutations, D128Y, G443R, and G614V, were undetectable in 100 chromosomes from healthy Italian controls and the amino-acid alignment of c-Mpl orthologs showed conservation of these three residues from *Homo sapiens to Bos taurus*, suggesting that they may exert a fundamental role in receptor structure and function (Figure 1C).

Low platelet c-MPL and high serum TPO

Whereas platelet surface expression of GPIbα, GPIX, GPIV, GPIIb, GPIIIa and GPIa was normal in all investigated subjects (*data not shown*), *c-MPL* was scarcely detectable in all CAMT patients, while its level was intermediate between that of patients and controls in heterozygous subjects (Figure 2 and Table 2). Consistently with the model of TPO regulation via ligand-induced internalization of c-MPL, serum TPO concentrations were greatly increased in patients as compared to controls. The serum TPO level was slightly elevated also in some carriers. However, no obvious correlation was observed between the type of mutation (missense and nonsense or frameshift) and the entity of c-MPL and TPO abnormalities either in patients or heterozygous individuals. Moreover, the D128Y mutation was associated with different TPO and c-MPL levels in the parents of patient CAMT1.

Reduced bone marrow colony-forming capacity

Colony-forming unit assays were performed before (patients CAMT1 and CAMT2) and/or after the development of trilineage bone marrow failure (patients CAMT2, CAMT3, and CAMT4). In all investigated patients CFU-Mk were either absent or severely reduced, while BFU-E and CFU-GM ranged from absent to values at the lower levels of normal ranges (Table 1). In patient CAMT2, while some BFU-E and CFU-GM were observed before the

Table 2. *c-MPL* mutations, platelet surface expression of c-Mpl and serum TPO level in CAMT patients and their relatives.

	Mutated protein variants[a] Mutated alleles	*Platelet c-MPL[b] (ratio anti-cMPL/ anti-isotype)*	*Serum TPO[c] (pg/mL)*
CAMT1	**D128Y/D128Y c.382G>T/c.382G>T** (exon 3)	1.16	2729
Father	+/D128Y	1.48	128
Mother	+/D128Y	1.91	<7
Sister	+/D128Y	nd	<7
CAMT2	**G443R**/R43X **c.1327G>C**/c.127C>T (exon 9/exon 2)	1.09	2783
Father	+/G443R	1.8	84
Mother	No mutation identified[d]	2.17	<7
CAMT3	**Y63X/G614V c.189C>G/c.1841G>T** (exon 2/exon 12)	1.07	2329
Father	+/Y63X	1.41	<7
Mother	+/G614V	1.51	<7
CAMT4	L79EfsX84/**K553RfsX75** c.235-236delCT/**c.1653delG** (exon 3/exon 11)	1.28[e]	2510[e]
Father	+/c.235-236delCT	1.65	68
Mother	+/c.1653delG	1.87	<7
CAMT5	L79EfsX84/**K553RfsX75** c.235-236delCT/**c.1653delG** (exon 3/exon 11)	nd	nd
Father	+/c.235-236delCT	nd	nd
Mother	+/c.1653delG	nd	nd

[a]GenBank entry NM_005373 (the A of the ATG of the initiator Met codon is reported as nucleotide +1) and NP_005364 for mRNA and protein, respectively; novel mutations are indicated in bold; [b]Mean value of platelet c-MPL in 10 healthy subjects was 2.18±0.39; [c]Serum TPO in 20 healthy subjects was <7-14 pg/mL; [d]R43X is a de novo mutation not identified in the mother's DNA sample (see text); [e]Test performed during the aplastic phase, whereas in all other cases platelet c-MPL and serum TPO were measured in CAMT patients before bone marrow failure; nd: not done.

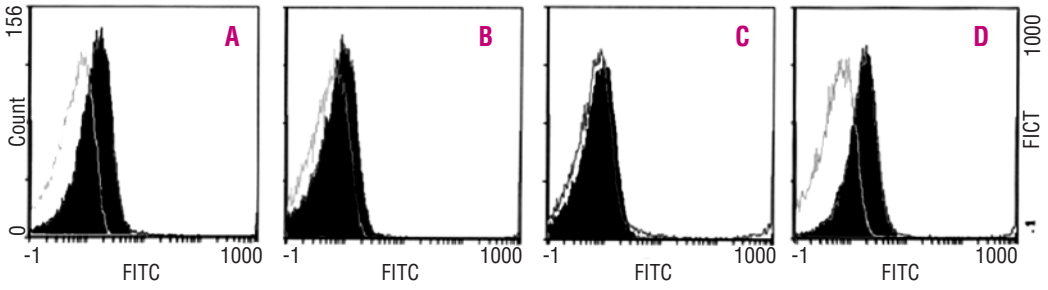

Figure 2. Tracings of flow cytometry for platelet c-MPL in CAMT1 family. Flow cytometric detection of c-MPL expression (solid areas) on the surface of platelets from control (A), heterozygous father (B), patient before (C) and after (D) hematopoietic stem cell transplantation. Tracings obtained with non-specific isotype control antibody are reported (open areas).

Table 3. Intracellular expression of TNF-α and IFN-γ in $CD3^+$ and $CD3^-$ bone marrow cells.

	TNF-α expression (MRFI/% positive cells)		*IFN-γ expression (MRFI/% positive cells)*	
	$CD3^+$	$CD3^-$	$CD3^+$	$CD3^-$
CAMT1	3.4/20	8/55	11.0/27	12.0/51
CAMT2	2.3/8	3.9/24	4.5/10	3.9/16
CAMT4	1.3/11	1.8/9	3.4/12	1.9/3
CAMT5	1.0/6	1.5/9	1.2/8	1.2/4
Controls*	1.8±0.3/2.3±1.8	1.9±0.5/3.9±2.6	2.2±0.6/5.5±2.1	1.9±0.6/4.4±2.8

*MRFI: mean relative fluorescence intensity. *Normal values in ten healthy subjects.*

development of bone marrow failure, they were completely absent during the aplastic phase. However, the highest numbers of the *in vitro* colonies were obtained in patients CAMT3 and CAMT4 during aplasia. Thus, there was no clear correlation between colony assay and the clinical course.

High levels of intracellular cytokine expression

During the aplastic phase all patients but CAMT3 were assessed for intracellular expression of TNF-α and IFN-γ in marrow $CD3^+$ and $CD3^-$ cells. Figure 3 is a scattergram of the intracytoplasmic expression of cytokines in patient CAMT1. Overall, all patients showed increased expression of intracytoplasmic TNF-α and IFN-γ in marrow cells and there seems to be a fair concordance between the two expression systems. In particular (Table 3) patients CAMT1 and CAMT2 displayed increased amounts of these cytokines in $CD3^+$ and $CD3^-$ marrow cells both in terms of percentage of positive cells and MRFI. Patient CAMT4 had increased expression of TNF-α in $CD3^+$ and $CD3^-$ marrow cells only when the percentage of positive cells was considered. IFN-γ was over-expressed only in $CD3^+$ cells both in terms of percentage and MRFI. Patient CAMT5 showed increased TNF-α in $CD3^+$ and $CD3^-$ cells as percentages and of IFN-γ only in $CD3^+$ in terms of percentages of cells.

Furthermore, patients CAMT1 and CAMT2, who had the highest expression of myelosuppressive cytokines, showed the lowest number of committed progenitors. Since patients were infection-free for at least 4 weeks before and 2 weeks after testing, the TNF-α and IFN-γ increases cannot be attributed to infections preceding or at the time of testing and should be regarded as a new biological finding in the aplastic evolution of CAMT.

Discussion

CAMT is a rare genetic disease with a limited numbers of cases reported in literature since the cloning of the gene responsible.[1,4-11] However, the diagnosis of five patients in two centers during the last 2 years raises the suspicion that the incidence of the disease is underestimated, presumably because the initial presentation of CAMT with isolated thrombocytopenia can be easily misdiagnosed with idiopathic thrombocytopenic purpura, while the late pancytopenic phase is indistinguishable from aplastic anemia. Thus, bone marrow examination should be part of the diagnostic work-up in all children with severe thrombocytopenia since birth and screening of the *c-MPL* gene should be performed when a reduced number of megakaryocytes is observed.

Consistent with a wide spectrum of *c-MPL* mutations, we identified three missense, two nonsense and two frame-shift mutations, five of which were novel alterations. Of note, to the best of our knowledge, the mutation in patient CAMT2 is the first reported *de novo* mutation in the *c-MPL* gene leading to CAMT, an autosomal recessive disease in which, by definition, parents should carry one mutated allele. However, now that it possible to verify the carrier state, an increasing number of cases, including patients with cystic fibrosis, spinal muscular atrophy and steroid 21-hydroxylase deficiency, appear to be important, even if rare, exceptions to this rule.[19] Recently, Germeshausen *et al.*,[9] who studied genotype/phenotype correlations in 23 patients, concluded that genotypes homozygous for nonsense or frame shift mutations, which are expected to result in loss of c-MPL expression, caused a severe form of the disease (named CAMT I) characterized by persistently low platelet counts and early progression to bone marrow aplasia. By contrast, genotypes homozygous for missense mutations, expected to maintain residual activity of c-MPL, induced a milder form (CAMT II) with transient increases of platelet counts over 50×10^9/L during the first year of life and later development of pancytopenia. Information from genotype/phenotype correlations could help clinicians decide the management of patients, for instance by enabling identification of the optimal timing at which hematopoietic stem cell transplantation, the only therapeutic intervention able to definitively cure CAMT patients, should be offered. However, there was no evidence of genotype/phenotype correlations in our study. All patients developed pancytopenia at an age comprised between 22 and 49 months (Table 1). Pancytopenia developed earliest in a 22-month old patient (CAMT3), a compound heterozygote for a missense and a nonsense mutation, while the longest aplasia-free period (49 months) was observed in a patient (CAMT4) carrying two frameshift

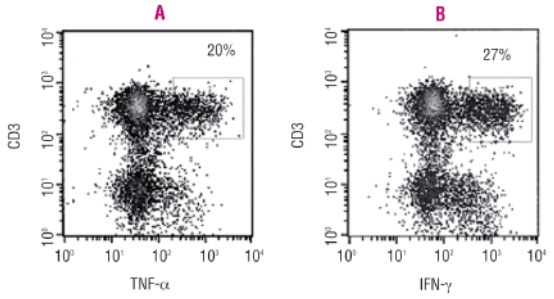

Figure 3. Flow cytometry for intracellular expression of TNF-α and IFN-γ. The scattergram represents $CD3^+/TNF\alpha^+$ (A) and $CD3^+/IFN\ \gamma^+$ (B) bone marrow cells of patient CAMT1. Squares include double positive cells. In the top right corner of each scattergram the percentage of double positive cells is reported.

mutations. Moreover, the only patient homozygous for a missense mutation developed severe pancytopenia at 38 months of age. Although the small number of investigated patients and the absence in our cases series of subjects with no c-MPL expression do not allow us to conclude that the genotype/phenotype correlations described previously do not exist, we suggest that early bone marrow failure is not exclusive to patients with mutations predicting severe defects of the TPO receptor, but can also affect patients with missense mutations.

We did not find any correlation even between the type of mutation and the expression of c-MPL on the platelet surface, as all patients, independently of whether their mutations were amino acid substitutions or truncated proteins, showed severely reduced levels of expression. Consistently with these results, TPO serum levels were similarly high in all patients. The TPO receptor is a transmembrane protein of 635 amino acids without intrinsic tyrosine kinase activity, containing four functional domains, a signal peptide (1-25 residues), an extracellular domain (26-491 residues) consisting of two cytokine receptor domains, a transmembrane domain (492-513 residues), and an intracellular domain (514-635 residues), necessary for ligand-induced proliferation and differentiation.[20] Therefore, a prediction of the effect of the mutations should take into consideration where proteins are mutated. For instance, since the c.1653delG allele is expected to code for a c-MPL protein (K553RfsX75) retaining the transmembrane domain, the receptor might be able to localize on platelet membrane. On the other end, alleles carrying the R43X, Y63X, and F126LfsX5 mutations are likely not to be expressed on the platelet surface because of the premature truncation of the receptor in the NH_2-terminus. As regards the missense mutations D128Y, G443R, and G614V, even if they occur at residues conserved through evolution, neither qualitative nor quantitative defects of the TPO receptor can be predicted since they are located in regions of poorly defined functional significance. Our data on c-MPL expression obtained by flow cytometry seem to indicate that all mutations we identified prevented or very severely affected membrane localization of this receptor. However, these results must be interpreted with caution, since flow cytometry is a semiquantitative method and its sensitivity is low when, as for c-MPL, the density of the investigated epitope approaches the limit of detection. Therefore, it would be of fundamental importance, before performing any genotype-phenotype analysis, to evaluate the effect of mutations using *in vitro* assays to test the expression levels and functional activity, at least in terms of TPO binding capacity, of the mutated proteins. Moreover, it would also be important to investigate only homozygous subjects to avoid misinterpretations, since the co-inheritance of two different types of mutations could influence the TPO receptor levels in different ways. It has been suggested that measuring TPO reactivity of platelets (potentiation of platelet activation induced by ADP) and bone marrow progenitors (increased *in vitro* colony formation under SCF, IL-3 and IL-6 stimulation) has a prognostic significance, as it distinguishes patients without any c-MPL activity from those with residual activity of the receptor.[5,9] However, also the tests for evaluating the effect of TPO on hemopoietic colony growth and the priming effect of TPO on ADP-induced platelet activation should be interpreted with caution, because they measure subtle differences in complex biological processes, and they do not permit reliable decisions to be made, such as choosing the time to perform hematopoietic stem cell transplantation.

Our observation that a few CFU-Mk were obtained in three patients (CAMT2, CAMT3, and CAMT4) under TPO stimulation 6 months before or during bone marrow failure supports this conclusion. Hematopoiesis is a complicated process regulated by a number of interconnected stimulatory and inhibitory pathways. Thus, many factors are likely to modulate the biological effects of an altered TPO receptor with different consequences on the clinical course of the disease in different patients. Still unknown age-related changes in this complex network could explain why erythroid and myeloid precursors decline in CAMT during aging leading to severe bone marrow aplasia. The increased expression of myelosuppresive cytokines documented for the first time in our patients might be involved in this process.

Transfusions have been blamed for cytokine increases, and indeed our patients had received some platelet concentrates before being investigated. However, the majority of already published data shows that TNF-α and IFN-γ overexpression is not related to transfusions,[21,22] and therefore it is reasonable to assume that the inhibitory cytokine increase we observed was part of the disease. TNF-α and IFN-γ act as late mediators of hematopoietic stem cell damage in acquired aplastic anemia,[21,23] an autoimmune disease in which autoreactive T cells damage hematopoietic cells. The observation that in our CAMT patients TNF-α and IFN-γ were over-expressed also in $CD3^-$ marrow cells, including monocytes and myelo-erythroid progenitors that are not usually involved in autoimmunity, suggests that these cytokines do not operate via an autoimmune mecha-

nism in this disease. Shortage of cells due to the aplastic phase in which CAMT patients were studied precluded the performance of further experiments. Therefore some aspects, such as the link between the *c-MPL* mutation and cytokine expression or the true myelosuppressive potential of these agents, could not be addressed. However, elevated intracellular levels of IFN-γ and TNF-α have also been detected in the bone marrow of patients with Fanconi anemia, another genetic, non-immune-mediated marrow failure disease.[18] Given that the increased amounts of myelosuppressive cytokines we found in CAMT subjects were of the same magnitude as those seen in Fanconi anemia[18] and in acquired aplastic anemia,[21] in which these effectors were proven to be contributory to marrow suppression, it can be speculated that also in CAMT TNF-α and IFN-γ might have had a role in the development of marrow failure. In conclusion, we identified new mutations responsible for CAMT and provide new insights into the possible pathogenic mechanism of marrow failure. In addition, we demonstrated that the previously suggested correlation between genotype and the time course of bone marrow failure is not reliable in all patients.[9] Thus, our study indicates that the natural history of CAMT is not always predictable on the basis of current knowledge of the disorder and suggests that hematopoietic stem cell transplantation from an HLA-matched donor, either related or unrelated, should be considered as early as possible in all patients. Further investigations are required to better understand the biological consequences of *c-MPL* mutations and to identify efficient prognostic parameters to be used for guiding therapeutic decisions.

Author's contributions

FDB, SF, MDS and MS made genetic analysis; AC measured intracellular cytokines; PN and CA performed platelet flow cytometry and measured serum TPO levels; VR performed hematopoietic colony assay; FL, CD and MZ controlled and analyzed data; AS and CLB wrote the paper; and all authors checked the final version of the manuscript.

Conflict of Interest

The authors reported no potential conflicts of interest.

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