c-mpl mutations are the cause of congenital amegakaryocytic thrombocytopenia

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Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare disease presenting with isolated thrombocytopenia in infancy and developing into a pancytopenia in later childhood. Thrombopoietin (TPO) is the main regulator of thrombopoiesis and has also been demonstrated to be an important factor in early hematopoiesis. We analyzed 9 patients with CAMT for defects in TPO production and reactivity. We found high levels of TPO in the sera of all patients. However, platelets and hematopoietic progenitor cells of patients with CAMT did not show any reactivity to TPO, as measured by testing TPO-synergism to adenosine diphosphate in platelet activation or by megakaryocyte colony assays. Flow cytometric analysis revealed absent surface expression of the TPO receptor c-Mpl in 3 of 3 patients. Sequence analysis of the c-mpl gene revealed point mutations in 8 of 8 patients: We found frameshift or nonsense mutations that are predicted to result in a complete loss of c-Mpl function in 5 patients. Heterozygous or homozygous missense mutations predicted to lead to amino acid exchanges in the extracellular domain of the receptor were found in 3 other patients. The type of mutations correlated with the clinical course of the disease. We propose a defective c-Mpl expression due to c-mpl mutations as the cause for thrombocytopenia and progression into pancytopenia seen in patients with CAMT. (Blood. 2001;97:139-146)

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Introduction

Congenital amegakaryocytic thrombocytopenia (CAMT) is a rare disease characterized by a severe hypomegakaryocytic thrombocytopenia during the first years of life that develops into a pancytopenia in later childhood, suggesting a general defect in hematopoiesis. Bone marrow transplantation is the only curative therapy for CAMT so far.

The pathophysiology of CAMT is not well understood. Experiments of Freedman and Estrov demonstrated that the cause of CAMT seems to be an intrinsic stem cell defect rather than an abnormality of the bone marrow microenvironment or an inhibitory factor in the patients' plasma. Serum levels of thrombopoietin (TPO), the pivotal regulator of megakaryopoiesis but also an important factor for early multipotential hematopoietic progenitors, are highly elevated in patients affected from CAMT.

Recently, Muraoka and coworkers described a patient with CAMT who had a defective response to TPO in megakaryocyte colony formation, decreased numbers of erythroid and myeloid progenitors, and elevated TPO serum levels. Ihara and coworkers detected 2 heterozygous point mutations in the c-mpl gene of this patient that were predicted to result in a complete absence of functional c-Mpl protein.

In this report we describe a defective TPO reactivity of hematopoietic progenitor cells and platelets from 9 patients with CAMT. A lack of c-Mpl expression on the platelet surface could be demonstrated in 3 patients. Molecular sequencing of the c-mpl gene of 8 patients with CAMT revealed point mutations in all patients: We found frameshift or nonsense mutations in 5 patients belonging to a patient group with a very severe course of the disease, heterozygous or homozygous missense mutations were found in 3 other patients with a slower progression of the disease. We propose, that c-mpl mutations are the cause for the amegakaryocytic thrombocytopenia and the development of a pancytopenia in patients with CAMT. The type of mutations could be predictive for the course of the disease.

Patients, materials, and methods

Patients

Platelets and/or bone marrow mononuclear cells from 9 patients with CAMT were examined at different time points. All patients were found to be thrombocytopenic during the first year of life, had normal radii, and showed no or severely reduced numbers of megakaryocytes in bone marrow smears. Four of the patients are from Kurdish origin and have consanguineous parents. (Approval was obtained from the institutional review board for these studies. Informed consent was provided according to the Declaration of Helsinki.) Retrospective comparison of the clinical data from 18 patients with CAMT from different German clinics resulted in a division into 2 different groups of patients with a different time course of thrombocytopenia: About 60% of the patients presented with a more severe form of CAMT with an early development from isolated thrombocytopenia into pancytopenia (group I). A second group of patients demonstrated a transient increase of platelet counts during the first year of life and a later development of pancytopenia (group II). The patients’ characteristics with the division into the 2 patient groups are described in Table 1.
Table 1. Patients’ characteristics

<table>
<thead>
<tr>
<th>Patient identification</th>
<th>Sex</th>
<th>Age (y)</th>
<th>BMT (age) (y)</th>
<th>Patient group*</th>
<th>Consanguineous parents</th>
<th>WBC (nL⁻¹)</th>
<th>Hgb (g⋅dL⁻¹)</th>
<th>Plt (nL⁻¹)</th>
<th>Tests performed†</th>
</tr>
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<tbody>
<tr>
<td>CAMT-1</td>
<td>F</td>
<td>0.6</td>
<td>0.3</td>
<td>I</td>
<td>+</td>
<td>7.8</td>
<td>5.6</td>
<td>20</td>
<td>2, 6</td>
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<td>CAMT-6</td>
<td>F</td>
<td>0.8</td>
<td>0.7</td>
<td>I</td>
<td>–</td>
<td>10.3</td>
<td>11.5</td>
<td>6</td>
<td>1, 4</td>
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<td>CAMT-7 (died after BMT)</td>
<td>M</td>
<td>7.0</td>
<td>1.4</td>
<td>II</td>
<td>–</td>
<td>6.8</td>
<td>11.1</td>
<td>26</td>
<td>7</td>
</tr>
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<td>CAMT-8</td>
<td>M</td>
<td>2.8</td>
<td>3.4</td>
<td>II</td>
<td>–</td>
<td>12.4</td>
<td>11.1</td>
<td>14</td>
<td>1, 2, 4</td>
</tr>
<tr>
<td>CAMT-9</td>
<td>F</td>
<td>4.2</td>
<td>2.3</td>
<td>I</td>
<td>+</td>
<td>12.4</td>
<td>11.1</td>
<td>7</td>
<td>2, 4</td>
</tr>
<tr>
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<td>F</td>
<td>3.0</td>
<td>7.8</td>
<td>II</td>
<td>–</td>
<td>6.5</td>
<td>13.0</td>
<td>29</td>
<td>1, 2, 4, 5</td>
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<tr>
<td>CAMT-12</td>
<td>M</td>
<td>4.1</td>
<td>9.3</td>
<td>II</td>
<td>–</td>
<td>4.6</td>
<td>12.6</td>
<td>25</td>
<td>3, 4, 5</td>
</tr>
<tr>
<td>CAMT-13</td>
<td>F</td>
<td>3.8</td>
<td>3.7</td>
<td>I</td>
<td>+</td>
<td>8.3</td>
<td>11.7</td>
<td>7</td>
<td>1, 3, 4</td>
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<td>CAMT-17</td>
<td>F</td>
<td>1.9</td>
<td>1.5</td>
<td>I</td>
<td>+</td>
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<td>9.1</td>
<td>35</td>
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<tr>
<td></td>
<td></td>
<td>0.8</td>
<td></td>
<td></td>
<td></td>
<td>8.6</td>
<td>9.8</td>
<td>13</td>
<td>6</td>
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<td>1.8</td>
<td></td>
<td></td>
<td></td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>1, 2</td>
</tr>
</tbody>
</table>

BMT = bone marrow transplantation; WBC = white blood cells; Plt = platelets.

*See “Patient, materials, and methods.”
†Tests performed: 1. Thrombopoietin (TPO) enzyme-linked immunosorbent assay (ELISA); 2. TPO bioassay; 3. c-Mpl expression (flow cytometry); 4. TPO reactivity (flow cytometry); 5. tyrosine phosphorylation in platelets; 6. colony assay from bone marrow mononuclear cells (BM-MNCs); 7. sequencing of c-mpl gene.
‡Platelet transfusion.
na: data not available.

Materials

Recombinant human (rh) TPO and the polyclonal rabbit antibody against human c-Mpl were kindly provided by Dr A. Shimosaka, Kirin Brewery (Tokyo, Japan). Amgen (Thousand Oaks, CA) provided rh granulocyte colony-stimulating factor (G-CSF) and rh stem cell factor (SCF). The rh granulocyte-macrophage colony-stimulating factor (GM-CSF) and rh interleukin-3 (IL-3) were gifts from Behringwerke (Marburg, Germany); rh erythropoietin (EPO) was obtained from Boehringer Mannheim (Mannheim, Germany). Adenosine diphosphate, bovine pancreatic insulin, transferrin, prostaeglandin E1, acetyl salicylic acid, apyrase (type VII), and bovine serum albumin were purchased from Sigma (Deisenhofen, Germany). Monoclonal antibodies against CD62P (clone CLB-Thromb/6) and CD41 (clone P2) as well as the IgG1 isotype control were purchased from Coulter-Immunotech (Hamburg, Germany), the recombinant, peroxidase-conjugated, antiphosphotyrosine antibody (RC20) was from Transduction Laboratories (Lexington, KY), the polyclonal antibody against Jak2 was from Santa Cruz Biotechnology (Santa Cruz, CA). Cell culture media and fetal calf serum were purchased from Life Technologies (Eggenstein, Germany). Collagen solution was obtained from StemCell Technologies (Vancouver, British Columbia) and methyl cellulose (Methocel A4C) from WAK-Chemie (Bad Homburg, Germany).

Assays for colony-forming units

For detection of colony-forming unit (CFU)-GM and CFU/burst-forming unit, erythrocytes (BFU-E) we used a methyl-cellulose–based culture system. The 10⁵ bone marrow mononuclear cells (BM-MNCs) were cultured in 1 mL of a semisolid medium containing 0.7% methyl cellulose, 30% fetal bovine serum, and 0.5 × 10⁻⁹ M 2-mercaptopethanol in Iscove’s modified Dulbecco’s medium (IMDM) with the hematopoietic growth factors rhG-CSF, rhGM-CSF, rhIL-3, rhSCF (all 10 ng/mL), and rhEPO (1 U/mL). The cultures were incubated at 37°C in an atmosphere of 5% CO₂ and 100% humidity for 14 days. After this time, colonies consisting of more than 50 cells were classified according to their morphology and counted.

For detection of CFU-megakaryocytes (CFU-Mks), we used a serum-free, collagen-based culture system. The 10⁵ BM-MNCs were cultured in a semisolid medium containing bovine serum albumin (10 mg/mL), bovine pancreatic insulin (10 μg/mL), human transferrin (iron-saturated, 200 μg/mL), 2-mercaptoethanol (0.5 × 10⁻⁹ M), collagen (1.1 mg/mL) in IMDM. Two different growth factor combinations were used: (1) rhTPO alone (50 ng/mL) and (2) rhTPO (50 ng/mL), rhIL-3 (10 ng/mL), and rhIL-6 (10 ng/mL). After 10 to 12 days of culture (37°C, 5% CO₂, 100% humidity), collagen gels were dehydrated on slides and immunocytochemically stained with a primary monoclonal antibody against CD41 (clone P2) and an APAAP (alkaline phosphatase monoclonal antialkaline phosphatase) detection system. Colonies consisting of 5 or more CD41 positive cells were counted as CFU-Mk.

Thrombopoietin serum levels

Patients’ sera were collected and stored at ~80°C until analysis. The measurement of TPO was performed with either a commercially available capture enzyme-linked immunosorbent assay (ELISA) system (R&D systems, Abingdon, UK) or an ELISA system recently developed by Folman et al. Some of the samples were additionally tested in a TPO bioassay using the human c-mpl–transfected cell line 32D clone23 as recently described.11
Flow cytometric analysis of in vitro platelet activation

TPO reactivity of platelets was tested as already described. In brief, platelet-rich plasma (PRP) was preincubated for 1 minute with or without rhTPO (50 ng/mL) before the platelet activator ADP (5 μM) was added. The stimulation was stopped by adding 1 mL of a formaldehyde solution (1% in phosphate-buffered saline [PBS]). Unstimulated platelets serving as a negative control were fixed immediately after preparation of PRP. After fixation for 30 minutes on ice, the samples were washed and stained for flow cytometric analysis. FITC-conjugated mAb anti-P-selectin (CD62P) as an activation-dependent marker and phycoerythrin (PE)-conjugated anti-gpIIb/IIIa (CD41) as a pan-platelet marker were used for measuring platelet activation. FITC- and PE-labeled isotype control antibodies were used as controls. After staining, the platelets were analyzed in a FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany).

Flow cytometric analysis of c-Mpl expression on platelets

We used a polyclonal rabbit antiserum against human c-Mpl for flow cytometric detection of c-Mpl on the platelet surface. Incubation of unfixed platelets with the primary antibodies was followed by the staining with the FITC-conjugated goat-antirabbit Ig (Dako, Glostrup, Denmark) and analysis on a FACScan flow cytometer.

Western blot detection of tyrosine phosphorylation in platelets

PRP was preincubated with acetyl salicylic acid (2 mM) for 30 minutes at room temperature. Prostaglandin E1 (1 μM) was added immediately before centrifugation of the platelets. The pellet was washed in a modified HEPES-Tyrode’s buffer containing appyrase (2 U/mL). For stimulation with rhTPO (2.7 μg/mL), platelets were resuspended in a concentration of 4 × 10^8/ml in the same buffer supplemented with 1 mM CaCl2. The stimulation of platelets was terminated by adding one volume of 2 × concentrated sample buffer (10% glycerol, 1% SDS, 5% 2-mercaptoethanol, 50 mM Tris, pH 6.8, 10 mM EGTA, and 1 mM Na3VO4 and 0.002% bromophenol blue). After SDS-gel electrophoresis proteins were transferred to a nitrocellulose membrane (0.45 μm). Tyrosine phosphorylated proteins were detected with the horseradish peroxidase (HRP)-conjugated recombinant antibody RC20 using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech, Freiburg, Germany) according to the manufacturer’s instructions. For immunoprecipitation, platelet stimulation was terminated by adding an equal amount of lysis buffer (15 mM Tris-HCl, 500 mM KCl, pH 8.3). Exons 5 to 8, 11, and 12 were amplified in 1× PCR buffer using 100 ng template DNA, 2 mM MgCl2, 0.1% Triton-X 100, 20 μM dNTP, and 25 pmol of each primer (exon 9: 5’TCTTTGTGGGAAATCTCCGAC, 5’AGGCCGCTGTCGGCGTTTGG, exon 10: 5’AGTATGCGGGCTGCTTATGTA, 5’GAGATCTGGGCTTGACAGA) in PCR buffer. All PCRs included 1 unit Taq DNA polymerase (Roche, Mannheim, Germany) and Pfu DNA polymerase (Stratagene, La Jolla, CA) in a total reaction volume of 25 μL. The PCRs were performed at 95°C for 5 minutes, followed by 35 cycles at 95°C for 1 minute, 58°C (exons 1-8, 11, 12) or 60°C (exons 9 and 10) for 1 minute, and 72°C for 1 minute, with a final extension at 72°C for 10 minutes. Direct sequencing of the purified PCR fragments was performed on a semiautomated sequencer (LI-COR, MWG-Biotech, Ebersberg, Germany) using the Thermo Sequenase cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Freiburg, Germany) and fluorescent c-mpl primers.

Results

Thrombopoietin serum levels

TPO serum levels were highly elevated in all patients (Table 2). No correlation between serum levels and platelet counts in the patients could be observed. Sera of 7 patients were tested for TPO bioactivity, all of them supported the growth of the factor-dependent, c-mpl–transfected cell line 32D clone 2310 (Table 2), excluding a not biologically active TPO as the cause for the thrombocytopenia.

Thrombopoietin reactivity of platelets

TPO synergizes with ADP in platelet activation. Preincubation with TPO enhanced the activating effect of ADP in platelets from healthy donors (Figure 1B) or from patients affected from immunologic thrombocytopenias (data not shown). In contrast, we were not able to detect any synergism between TPO and ADP (Figure 1A) in platelets from all patients with CAMT tested (10 samples from 7 patients, described in Table 1). The activation obtained with ADP alone in platelets from patients with CAMT was similar to that in healthy donors.

c-Mpl signaling in platelets of a patient with CAMT

c-Mpl is a member of the cytokine receptor superfamily and leads to tyrosine phosphorylation of cellular proteins after stimulation. We could demonstrate that stimulation of platelets from healthy donors

<p>| Table 2. Thrombopoietin serum levels in patients with congenital amegakaryocytic thrombocytopenia |</p>
<table>
<thead>
<tr>
<th>Patient identification</th>
<th>Age (y)</th>
<th>TPO serum level (pg/mL)</th>
<th>TPO bioactivity</th>
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<tbody>
<tr>
<td>CAMT-1</td>
<td>0.3</td>
<td>nt</td>
<td>+</td>
</tr>
<tr>
<td>CAMT-6</td>
<td>0.7</td>
<td>1606*</td>
<td>nt</td>
</tr>
<tr>
<td>CAMT-7</td>
<td>3.4</td>
<td>2653†</td>
<td>+</td>
</tr>
<tr>
<td>CAMT-8</td>
<td>6.5</td>
<td>3290*</td>
<td>nt</td>
</tr>
<tr>
<td>CAMT-9</td>
<td>2.2</td>
<td>2436*</td>
<td>+</td>
</tr>
<tr>
<td>CAMT-10</td>
<td>2.3</td>
<td>nt</td>
<td>+</td>
</tr>
<tr>
<td>CAMT-11</td>
<td>3.0</td>
<td>2083*</td>
<td>nt</td>
</tr>
<tr>
<td>CAMT-12</td>
<td>7.8</td>
<td>3226*</td>
<td>+</td>
</tr>
<tr>
<td>CAMT-13</td>
<td>3.7</td>
<td>1022*</td>
<td>nt</td>
</tr>
<tr>
<td>CAMT-14</td>
<td>1.5</td>
<td>2674†</td>
<td>+</td>
</tr>
<tr>
<td>CAMT-17</td>
<td>1.8</td>
<td>1092†</td>
<td>+</td>
</tr>
<tr>
<td>Healthy donors</td>
<td>(mean ± SD, n = 40)</td>
<td>120 ± 76</td>
<td>–</td>
</tr>
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</table>

*Measured with R&D systems enzyme-linked immunosorbent assay (ELISA).
†Measured with an ELISA system developed by Folman et al.9

DNA sequencing and mutation analysis

Genomic DNA was extracted from peripheral blood using standard procedures. Exons 1 to 4 of c-mpl gene were amplified by polymerase chain reaction (PCR) using 100 ng of template DNA, 1.5 mM MgCl2, 100 μM dNTP, and 10 pmol of each primer (exons 1-2: 5’CTGAAAGGGAGAGGATGGGG, 5’AGGGGACAGATCATGGG, exon 3: 5’GCATGGTGCCTGTGTAAGAG, 5’GTGCTATCCGGCGACCTGG, exon 4: 5’GACTGTTGACTCAGAG, 5’GGCGAAGTGAGAGTGGAG) in PCR buffer (100 mM Tris-HCl, 500 mM KCl, pH 8.3). Exons 5 to 8, 11, and 12 were amplified in 1× PCR buffer using 100 ng template DNA, 2 mM MgCl2, 0.1% Triton, 100 μM dNTP, and 10 pmol of each primer (exons 5-6: 5’TAGATTGGAAGCTTGGG, 5’CTCCCATGACACAAACC, exon 7-8: 5’GGGATTAGTCTCTTGAGGG, 5’CCTCCCTGCTTGGGGTCC, exons 11-12: 5’CCTCCGCTGACCC, 5’GTTAGGGTGAGCGATT) in PCR buffer. Exons 9 and 10 were amplified by PCR using 100 ng template DNA, 3 mM MgCl2,
donors with TPO led to phosphorylation of several proteins, the most prominent band appearing at about 95 kd (Figure 2A). We were able to investigate c-Mpl signaling in platelets from patient CAMT-11, which did not show any difference in the tyrosine phosphorylation pattern before and after stimulation with TPO (Figure 2A). The protein pattern of constitutively tyrosine-phosphorylated proteins between the healthy donor and the patient was similar, with the exception of a 60 kd protein band only detectable in the patient’s platelets, independent of the state of stimulation. This is most likely a consequence of a higher amount of contaminating plasma proteins in platelet preparations from thrombocytopenic patients. Subsequent analysis of TPO-dependent phosphorylation of Jak2 in platelets of patient CAMT-11 did not reveal any activation of this molecule in contrast to platelets of healthy donors, although Jak2 expression could be detected in this patient (Figure 2B).

Expression of c-Mpl in platelets

c-Mpl expression on the surface of platelets was analyzed in 3 patients. We used a polyclonal rabbit antiserum against c-Mpl. Specificity of the serum was tested with the c-mpl–transfected cell line 32D clone 23 (data not shown). The antibodies demonstrated a weak, but distinct reactivity with platelets from healthy donors (Figure 3B,D,F). In contrast, we could not detect any binding of the antibody to platelets from patients with CAMT (CAMT-7, -11, -12, Figure 3A,C,E).

Colony-forming unit assays

 Colony-forming assays were performed with bone marrow mononuclear cells of 5 patients, one of them at 2 different time points (Table 3). The growth of megakaryocytic and also of myeloid and erythroid colonies was impaired in all samples.

No growth of megakaryocytic colonies with TPO as a single growth factor could be observed, with the exception of one assay in which only 2 CFU-Mks grew. In contrast, TPO induced the growth of 42 ± 23 CFU-Mks per 10^6 BM-MNCs from healthy controls. We observed the growth of some megakaryocytic colonies after stimulation with IL-3, IL-6, and TPO only in samples from the younger patients with CAMT (less than 2 years). There was no growth of megakaryocytic colonies from samples of 2 patients older than 3 years.

In the younger patients, we found a strongly decreased growth

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**Figure 1. Costimulation of platelets with rhTPO, CAMT versus healthy control.** Platelets of patient CAMT-7 (A) and of a healthy control donor (B) were stimulated with ADP (5 μM) after preincubation with or without rhTPO (50 ng/mL). Flow cytometric analysis of platelet activation was performed using a monoclonal antibody against CD62P. CD62P expression of unstimulated platelets is shown in each histogram.

**Figure 2. Tyrosine phosphorylation in platelets after TPO stimulation.** Platelets of patient CAMT-11 and of a healthy donor were preincubated with 2.7 μg/mL rhTPO before lysis and Western blot analysis of tyrosine-phosphorylated proteins (A). Phosphorylation of Jak2 was proved after immunoprecipitation with anti-Jak2 and Western blot detection of tyrosine-phosphorylated proteins and Jak2 (B).

**Figure 3. Expression of c-Mpl on platelets of 2 patients affected from CAMT.** Flow cytometric detection of c-Mpl expression on the surface of platelets from patients CAMT-7 (A), CAMT-11 (C), and CAMT-12 (E) using a polyclonal antiserum against human c-Mpl. Because the flow cytometric measurements were made in different laboratories under slightly different conditions, individual controls from healthy donors are shown for each patient (B,D,F).
The c-mpl gene mutation analysis in patients with congenital amegakaryocytic thrombocytopenia

<table>
<thead>
<tr>
<th>Patient identification</th>
<th>Mutation*</th>
<th>Exon no.</th>
<th>Homozygous/heterozygous</th>
<th>Predicted aa sequence†</th>
<th>Inherited from</th>
<th>Patient group</th>
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<tr>
<td>CAMT-1</td>
<td>C127T</td>
<td>2</td>
<td>Homo-</td>
<td>A43X</td>
<td>Not tested</td>
<td>I</td>
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<tr>
<td>CAMT-6</td>
<td>C268T</td>
<td>3</td>
<td>Homo-</td>
<td>R90X</td>
<td>Father/mother</td>
<td>I</td>
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<td>G340A</td>
<td>3</td>
<td>Homo-</td>
<td>V114M</td>
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<td>I</td>
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<td>del378</td>
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<td>Frameshift: 126L WTVX</td>
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<td>I</td>
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<tr>
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<td>Homo-</td>
<td>79-161, stop after 161 aa</td>
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<td>CAMT-11</td>
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<td>2</td>
<td>Homo-</td>
<td>A43X</td>
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<td>I</td>
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<td>G305C</td>
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<td>Homo-</td>
<td>R102P</td>
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<td>CAMT-13</td>
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<td>3</td>
<td>Homo-</td>
<td>R102P</td>
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<td>CAMT-14</td>
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<td>3</td>
<td>Homo-</td>
<td>R102P</td>
<td>Father/mother</td>
<td>II</td>
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</tbody>
</table>

aa: amino acid(s).

*Although sequencing was performed on genomic DNA, the position of mutations are given as positions in c-mpl-P cDNA.

†Numbering of amino acids according Mpl-P sequence.

The c-mpl gene of patient CAMT-13 is characterized by the homozygous delCT at nucleotide positions 235/236 of c-mpl cDNA leading to an altered amino acid sequence from position 79 until a stop after amino acid 161. The mutations found in patients CAMT-1, -6, -9, -13, and -17 are predicted to lead to a complete loss of a functional TPO receptor mPl. All patients belong to the group I of patients with CAMT with a very severe form of CAMT with an early progression into pancytopenia.

Patients CAMT-7 and CAMT-12 demonstrated a homozygous G to C transversion in exon 3 of the c-mpl gene (G305C) that was inherited from their unrelated heterozygous parents and resulted in the substitution of the amino acid arginine by a proline at amino acid position 102. In patient CAMT-11, we found 2 heterozygous point mutations (C823A, G305C), both leading to amino acid exchanges (R102P, P275T) in the extracellular domain of C-Mpl. All patients with missense mutations belong to group II of patients with CAMT.
platelet activation has already been used as a diagnostic tool for the detection of defects in TPO reactivity.11,14,15 Because signal transduction pathways of the TPO receptor c-Mpl are essentially similar in platelets and their progenitors in the bone marrow, we assume a general defect of all c-Mpl–bearing cells in the hematopoietic system. The TPO reactivity of hematopoietic progenitors was tested in bone marrow samples from 5 patients. We were not able to detect growth of megakaryocytic colonies after stimulation with TPO as a single hematopoietic growth factor, with the exception of a very weak growth of CFU-Mks in a bone marrow sample from patient CAMT-7 at the age of 1.4 years. Examination of a later bone marrow sample from this patient (age: 5.6 years) revealed no reactivity to TPO anymore. A defective response to TPO in megakaryocyte-colony formation was described in one patient with CAMT by Muraoka et al.6

The defective response of platelets and hematopoietic progenitors to TPO was confirmed by studies on the signal transduction of c-Mpl in platelets, which are assumed to be representative for c-Mpl signal transduction in megakaryocytes and their progenitors: We observed no induction of intracellular tyrosine phosphor- ylations in total platelet lysates or in Jak-2 immunoprecipitates, respectively, after stimulation with TPO in patient CAMT-11.

So far, these results very much resembled those obtained with platelets from patients suffering from thrombocytopenia with absent radii (TAR) in a previous study.11 However, we could demonstrate that c-Mpl expression on TAR patients’ platelets was normal,11 and others found no alterations in the c-mpl gene of TAR patients.16 These results led us to the hypothesis that a defect in c-Mpl-dependent signaling pathways is the cause for thrombocytopenia in TAR syndrome.

To analyze the TPO receptor expression in patients with CAMT, we investigated the c-Mpl protein and the genomic sequence of the c-mpl gene. We could not detect c-Mpl on the surface of the platelets from 2 patients affected from CAMT. Sequence analysis revealed point mutations in 8 of 8 patients. Homozygous deletions (CAMT-9 and -13) or nonsense mutations (CAMT-1, -6, and -17) were found in 5 patients, all of them predicted to result, if translated, in a premature terminated c-Mpl protein, which lacks a transmembrane and intracellular domain. These patients should have a complete loss of c-Mpl function. Interestingly, all these patients belong to the group I of patients with CAMT who have a more severe thrombocytopenia and reveal a more rapid progression into pancytopenia. In contrast, patients CAMT-7 and CAMT-12 who demonstrated a homozygous missense mutation, and CAMT-11 with 2 heterozygous missense mutations in the c-mpl gene belong to group II. This might correspond to a residual function of c-Mpl in these patients.

The homozygous point mutation in exon 3 of c-mpl found in patients CAMT-7 and -12 leads to an exchange of arginine 102 to proline. This substitution, which is located close to the highly conserved cysteine residues, may have an important influence on protein folding and therefore on the binding of TPO. The receptor could not be detected on platelets of patients CAMT-7 and CAMT-12 by flow cytometry using a polyclonal antiserum against the extracellular domain of the protein. However, there might be a residual signal transduction activity of c-Mpl in these patients as could be deduced from the weak reactivity to TPO in a colony assay (CAMT-7) and from the fact that these patients were clinically stable over several years before the deteriorating hematopoiesis necessitated a bone marrow transplantation.

In patient CAMT-11, we detected 2 heterozygous missense mutation in the c-mpl gene that are inherited from her heterozygous parents. This patient presented with a less severe form of CAMT, she is the only one of the patients in this report who did not show a tendency for a pancytopenia to develop so far. CAMT-11 is homozygous for the mutation G305C, which causes a more severe course of the disease if homozygously expressed (CAMT-7, -12). We conclude that the exchange of proline to tyrosine at amino acid position 275, caused by the other heterozygous mutation found in this patient (C823A), seems to have less effect on c-Mpl function.

Mutations in the c-mpl gene in patients with CAMT have been reported by others: Ihara and coworkers’ detected 2 heterozygous point mutations in the c-mpl gene that were predicted to result in a
complete lack of functional c-Mpl protein in this patient. Van den Oudenrijn et al reported c-mpl mutations in 4 of 5 patients with CAMT, 3 of them with 2 heterozygous mutations, one with a homozygous splice defect. In contrast to the previous reported mutations in the c-mpl gene of patients with CAMT, which are evenly distributed over the c-mpl gene, we found a strong accumulation of mutations in exon 3, all mutations are located in the first cytokine receptor homology domain. The high frequency of homozygous mutations in our patients is partly due to the consanguinity in their patients (patients CAMT-9, -13, and -17). The mutation found in patients CAMT-7, -11, and -12 was reported for another patient by van den Oudenrijn et al. Because all these patients are unrelated and their parents are not consanguineous, this mutation seems to occur more frequently, at least in Europe.

There are some striking similarities in the phenotypes observed in patients affected from CAMT with c-Mpl-deficient mice generated by gene targeting. Disruption of the c-mpl gene in mice resulted in a specific loss of both megakaryocytes and platelets with an approximately 85% decrease in peripheral platelet counts. The remaining platelets were functionally normal.18 These observations correspond well to those made in patients with CAMT: We found very low platelet counts, as low as 5 x 10⁹/L, and these platelets had a normal reactivity to the platelet agonists TRAP or ADP. High TPO serum levels were demonstrated in c-mpl−/− mice.18 Because TPO knock-out mice demonstrate a very similar phenotype to the c-mpl−/−,19,20 a TPO production defect could also be postulated as a cause of CAMT. However, TPO serum levels have been shown to be generally high in these patients.5,19,21 In all patients tested so far, serum TPO revealed full biologic activity.5

In addition to the lineage specific effects on megakaryocypoi-tesis, there are deficiencies in other nonmegakaryocytic lineages of hematopoiesis in patients with CAMT.1,16 which also seem to be quite similar in c-mpl−/− mice.19,22,23 The role of TPO in early hematopoiesis has been elucidated by several investigators during the last few years. In vitro studies demonstrated that TPO supports the survival of multilineage progenitors or synergizes with other hematopoietic growth factors in expansion of these cells.24-28 Solar et al found a significantly better engraftment of CD34⁺CD38⁻/ c-Mpl⁺ human hematopoietic progenitor cells compared with CD34⁺CD38⁻/ c-Mpl⁻ cells in transplanting SCID mice.

Although no decline in peripheral blood counts in c-mpl−/− mice has been described, the impairment of nonmegakaryocytic progenitors in c-mpl−/− mice seems to deteriorate during ontogeny: Alexander et al demonstrated normal growth of hematopoietic progenitors of all lineages from the fetal liver of c-mpl−/− mice (day 12 of gestation), whereas growth of myeloid and erythroid progenitors as well as that of early bipotential or multipotential progenitors from hematopoietic tissues from neonate mice was already markedly reduced. In analogy, Freedman and Estrov demonstrated a decline in the growth of nonmegakaryocytic progenitors and earlier multipotential progenitors during the development from isolated thrombocytopenia into aplastic anemia in serial analyses of one patients with CAMT. In accordance with these observations, we found an age-dependent decline in the growth of nonmegakaryocytic progenitors with an earlier drop in the erythroid colonies. The decline in the growth of nonmegakaryocytic progenitors precedes the drop of the corresponding mature cells in the peripheral blood and may reflect an exhaustion of early multipotential progenitors due to the lack of TPO reactivity. Interestingly, we found growth of megakaryocyte colonies from BM-MNCs from 2 younger patients with CAMT using TPO, IL-3, and IL-6, but not using TPO as a single growth factor. This observation also supports the hypothesis that only the response to TPO is affected in CAMT. Several attempts have been made to treat patients with CAMT with hematopoietic growth factors: In a clinical study with rhIL-3 in 5 patients with CAMT, Guinan and coworkers could demonstrate at least a short-term improvement in platelet counts, bleeding diatheses, or transfusion requirement. A subsequent study with the GM-CSF/IL-3 fusion protein PIXY321 revealed a platelet count elevation only for those patients who demonstrated a modest impairment of hematopoiesis, suggesting that the early multilineage pool, which seems to be affected from the c-mpl defect, was not yet completely exhausted in these patients.20 Two of our patients (CAMT-7 and CAMT-9) were treated with rhIL-11 (NEUMEGA). After a short decrease of bleeding symptoms, platelet counts dropped under the baseline values from before therapy, which was stopped on day 10, and more than 6 weeks were needed for a complete platelet recovery. We conclude that rhIL-11 treatment led to a transient exhaustion of the megakaryocytic progenitor cell pool as an effect of faster maturation of megakaryocytes, which is followed by a decreased platelet counts for some weeks (G. Strauss, unpublished results).

In conclusion, we propose a defective c-Mpl expression leading to a defect in the reactivity to TPO as the cause for CAMT in the majority of patients. CAMT could be considered as a model for investigating the in vivo role of TPO in human hematopoiesis. From our observations, we may offer 2 hypotheses. The first one is that TPO and c-Mpl seem to be essential for growth and maintenance of multipotential hematopoietic progenitor cells. A lack or misfunction of c-Mpl leads to an exhaustion of the early progenitor cell pool over the first years of life. The second hypothesis is that mechanisms independent of c-Mpl are responsible for the formation of multipotential progenitors in early ontogeny. The understanding of these mechanisms might provide closer insights into hematopoietic regulation and a therapeutic approach in the treatment of CAMT.

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**c-mpl** mutations are the cause of congenital amegakaryocytic thrombocytopenia

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