NPC1 defect results in abnormal platelet formation and function: studies in Niemann–Pick disease type C1 patients and zebrafish

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Niemann–Pick type C is a lysosomal storage disease associated with mutations in NPC1 or NPC2, resulting in an accumulation of cholesterol in the endosomal–lysosomal system. Niemann–Pick type C has a clinical spectrum that ranges from a neonatal rapidly fatal disorder to an adult-onset chronic neurodegenerative disease combined with remarkably, in some cases, hematological defects such as thrombocytopenia, anemia and petechial rash. A role of NPC1 in hematopoiesis was never shown. Here, we describe platelet function abnormalities in three unrelated patients with a proven genetic and biochemical NPC1 defect. Their platelets have reduced aggregations, P-selectin expression and ATP secretions that are compatible with the observed abnormal alpha and reduced dense granules as studied by electron microscopy and CD63 staining after platelet spreading. Their blood counts were normal. NPC1 expression was shown in platelets and megakaryocytes (MKs). In vitro differentiated MKs from NPC1 patients exhibit hyperproliferation of immature MKs with different CD63+ granules and abnormal cellular accumulation of cholesterol as shown by filipin stainings. The role of NPC1 in megakaryopoiesis was further studied using zebrafish with GFP-labeled thrombocytes or DsRed-labeled erythrocytes. NPC1 depletion in zebrafish resulted in increased cell death in the brain and abnormal cellular accumulation of filipin. NPC1-depleted embryos presented with thrombocytopenia and mild anemia as studied by flow cytometry and real-time QPCR for specific blood cell markers. In conclusion, this is the first report, showing a role of NPC1 in platelet function and formation but further studies are needed to define how cholesterol storage interferes with these processes.

INTRODUCTION

Niemann–Pick diseases are autosomal recessive defects in sphingomyelin/cholesterol metabolism and belong to the larger family of lysosomal storage diseases. NP diseases types A and B are primary defects in acid sphingomyelinase activity, while NP diseases type C (C1 and C2) have defective intracellular processing of low-density lipoprotein-derived cholesterol, resulting in an accumulation of unesterified cholesterol in the endosomal–lysosomal system (1,2). NP type C has a prevalence of ~1 in 100 000 live births in western countries (1,2). Ninety-five percent of the patients have mutations in the NPC1 gene (MIM257220), while the remaining 5% have mutations in the NPC2 gene (MIM607625) (1–3). Both genes encode for endosomal–lysosomal proteins with NPC1 being a transmembrane protein and NPC2 a soluble protein (4). The exact functions of the NPC1 and NPC2 proteins are still unclear. NPC is currently considered a cellular cholesterol trafficking defect but in the brain, the prominently stored lipids are gangliosides.

The clinical spectrum of NPC ranges from a neonatal rapidly fatal disorder to an adult-onset chronic neurodegenerative disease (5,6). The neurological involvement defines the disease severity in most patients but is typically preceded by...
systemic signs as cholestatic jaundice in the neonatal period or isolated spleno- or hepatosplenomegaly in infancy or childhood. Loss-of-function mutations in the NPC1 gene lead to a failure of the calcium-mediated fusion of endosomes with lysosomes, resulting in the accumulation of cholesterol and other lipids in late endosomes and lysosomes (7). The initial laboratory diagnosis requires skin fibroblasts to demonstrate accumulation of unesterified cholesterol in lysosomes after staining with filipin (8). Pronounced abnormalities are observed in ~80% of the cases, while mild to moderate alterations in the remainder. Genotyping confirms the diagnosis and is essential for prenatal diagnosis. Symptomatic management of patients is crucial and a first product, Miglustat, has been granted marketing authorization in Europe and several other countries for specific treatment of the neurological manifestations (9).

Interestingly, in 1971, a report described for the first time coagulation and platelet changes in a NP disease patient (10). Thrombocytopenia, anemia and petechial rash were also described immediately after birth in patients with fetal onset NPC (5). NPC1 mutant mice in a C57BL/6J background presented with red blood cell abnormalities and abundant ghost erythrocytes in addition to abnormalities in white cells, such as cytoplasmic granulation and neutrophil hypersegmentation, that included lymphopenia and atypias (11). Remarkably, these blood cell abnormalities were not present when this NPC1 mutant was present in the original BALB/c background. It was also suggested that NPC2 plays a role in hematopoiesis as this protein, in the presence of thrombopoietin (TPO), increased human multipotent myeloid progenitor cells (CFU-GEMM) and decreased granulocyte–macrophage progenitors (CFU-GM) (12). A hematopoietic defect in NPC2 deficient patients has never been described probably because other molecules in the TPO system compensate for the loss of NPC2. Very recently, thrombocytopenia was also reported in most patients with NP disease type B, while anemia and leucopenia were less common in these patients (13). To our knowledge, there are no detailed studies on the role of NPC1 in megakaryopoiesis and/or platelet function in NPC1-deficient patients or under normal physiological conditions.

Platelets are easy to isolate in a non-active state and are ideal cells to evaluate signal transduction, secretion and adhesion in hemostatic disorders, but we have shown that these kinds of functional platelet studies are also useful to characterize the unknown role of a protein that is involved in a well-known disorder that includes only a mild clinical or even subclinical platelet defect (14,15). For example, we have found that Duchenne muscular dystrophy patients with dystrophin mutations have an impaired collagen platelet activation and a disorganized cytoskeletal platelet membrane organization that accounts for their increased blood loss during spinal surgery without generating a spontaneous platelet-related bleeding phenotype in these patients (16). We have therefore performed different functional platelet tests in three unrelated NPC1 patients, including one with a prolonged Ivy bleeding time. The morphology and functional activity of their platelets were studied in detail. NPC1 expression studies were performed in megakaryocyte (MK) cell lines and primary cells. The effect of NPC1 was also studied during the in vitro differentiation of peripheral blood cell-derived hematopoietic stem cells (HSCs) from patients versus control into MKs and early erythroblasts. Evidence for a role of NPC1 in platelet and erythrocyte formation was further addressed in transgenic zebrafish models after NPC1 depletion.

RESULTS
Clinical description of three unrelated NPC1 patients

Patient 1 is a girl who presented at the age of 10 days with cholestasis, which improved spontaneously and disappeared around at the age of 6 months, and with hepatosplenomegaly. Her length and weight followed the third percentile. Head circumference followed between the 10th and 25th percentiles. Developmental delay was apparent from the age of 18 months. At the age of 2 years, ptosis and internal strabismus were noticed. From the age of 26 months, there was neurological regression, with the loss of motor and cognitive milestones. Tube feeding was started. Developmental age was 10 months at the age of 31 months. There was progressive appearance of pyramidal dysfunction with spasticity, as well as anterior horn dysfunction with muscular atrophy and tongue fasciculations. EMG showed an axonal polyneuropathy and cerebral MRI showed progressive cortical atrophy and periventricular white matter changes. She died at the age of 3.5 years after further neurological and general deterioration. Studies in cultured skin fibroblasts showed strong reduction in exogenous cholesterol from lipoprotein, confirming the diagnosis of Niemann–Pick type C (Table 1) (17). Compound heterozygosity for two mutations in NPC1, S425X (paternal) and p.231Vfs (maternal), was found.

Patient 2 is a girl who presented on day 14 with cholestasis, which improved spontaneously, and hepatosplenomegaly. Electron microscopy of a liver biopsy at the age of 15 months showed lysosomal myelin-like inclusions in the hepatocytes and Kupffer cells. Mild developmental delay was apparent from the age of 18 months. From the age of 47 months, neurological regression, starting with vertical supranuclear gaze palsy, was noted. Cerebral MRI at that time was normal. In the following months, progressive supranuclear gaze palsy, ataxia, spasticity, kataplexia and mental deterioration became apparent, and therapy with Miglustat was started at the age of 5.5 years. The SARA score for ataxia was 18 at that time, and has remained stable until now (age 7.5 years). Studies in cultured skin fibroblasts that quantified the exogenous lipoprotein-derived cholesterol confirmed the diagnosis of Niemann–Pick type C (Table 1). Compound heterozygosity for the frequent I1061T mutation and a N169K mutation in NPC1 was found.

Patient 3 is a boy who presented in the neonatal period with cholestasis, which improved spontaneously, hepatosplenomegaly and bilateral inguinal hernia. Growth and development have been normal (current age is 6 years). Electron microscopy of a liver biopsy at the age of 3 months showed lysosomal myelin-like inclusions in the hepatocytes and Kupffer cells. Biochemical studies in cultured skin fibroblasts confirmed the diagnosis of Niemann–Pick type C (Table 1). Heterozygosity for the frequent I1061T mutation in NPC1 was found. Both parents are carriers.
### Table 1. Patients’ characteristics: NPC1 mutations, hematological counts and functional platelet studies

<table>
<thead>
<tr>
<th>Normal values</th>
<th>Hematological counts</th>
<th>Functional platelet tests</th>
<th>Electron microscopy</th>
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<tr>
<td></td>
<td>WBC</td>
<td>RBC</td>
<td>MCV</td>
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**Patients**

<table>
<thead>
<tr>
<th>Age (years); gender</th>
<th>NPC1 mutationsa</th>
<th>Cholesterol 3H-oleate (16)a</th>
<th>2950 ± 1200 pmol/mg</th>
<th>WBC</th>
<th>RBC</th>
<th>MCV</th>
<th>PLT</th>
<th>MPV</th>
<th>Aggregation epinephrine, 1.25 μM</th>
<th>Aggregation collagen, 2 μg/ml (0.5 μg/ml)</th>
<th>Aggregation ADP, 10 μM</th>
<th>ATP secretion collagen, 2 μg/ml</th>
<th>ATP secretion ADP, 10 μM</th>
<th>Electron microscopy</th>
</tr>
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<tbody>
<tr>
<td>Patient 1 Died</td>
<td>S425X V231fs</td>
<td>75 pmol/mg</td>
<td>10.1</td>
<td>12.5</td>
<td>4.6</td>
<td>74</td>
<td>77</td>
<td>159</td>
<td>122</td>
<td>10.0</td>
<td>20b</td>
<td>65 (52)</td>
<td>60</td>
<td>0.5</td>
</tr>
<tr>
<td>3.5; F</td>
<td></td>
<td></td>
<td>10.1</td>
<td>12.5</td>
<td>4.6</td>
<td>74</td>
<td>77</td>
<td>159</td>
<td>122</td>
<td>10.0</td>
<td>20b</td>
<td>65 (52)</td>
<td>60</td>
<td>0.5</td>
</tr>
<tr>
<td>Patient 2 8; F</td>
<td>I1061T N169K</td>
<td>&lt;10 pmol/mg</td>
<td>8.4</td>
<td>9.8</td>
<td>5.2</td>
<td>73</td>
<td>66.5</td>
<td>177</td>
<td>271</td>
<td>10.0</td>
<td>24b</td>
<td>56 (28)</td>
<td>55</td>
<td>0.28b</td>
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<tr>
<td>Patient 3 7; M</td>
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<td>30 pmol/mg</td>
<td>5.8</td>
<td>8.0</td>
<td>4.6</td>
<td>78</td>
<td>141</td>
<td>187</td>
<td>7.8</td>
<td>8.6</td>
<td>34b</td>
<td>58 (39)</td>
<td>43b</td>
<td>0.24b</td>
</tr>
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WBC, white blood cells; RBC, red blood cells; MCV, mean corpuscular volume; PLT, platelets; MPV, mean platelet volume.
aPerformed by Dr M.T. Vanier.
bz-Score ± 2.
Morphological and functional platelet studies in NPC1 patients

Interestingly, in addition to her other phenotypic features described above, patient 1 also presented with a prolonged ly bleeding time of 10 min. Her standard coagulation parameters were normal but functional platelet testing at multiple occasions showed reduced aggregation and secretion values. The three patients have in common a reduced aggregation response to low concentrations of Horm collagen and an absent secondary response to epinephrine (aggregation amplitudes are reported in Table 1). Aggregation to high doses of ADP and collagen were normal. These results are typically seen in platelets with secretion defects (18). Indeed, ADP secretion with collagen and especially ADP was reduced for the patients. Flow cytometry showed decreased expression levels of CD62P (P-selectin that is stored in platelet alpha granules) for patients (P2; 2760 and P3; 2563) versus control platelets (C1; 3696 and C2; 3548) after full activation with convulxin. No expression differences by flow cytometry were seen for the main platelet receptors GPIIla (CD61), GPIIb (CD41) and GPIb/IX (CD42a/CD42b). Electron microscopy showed a reduced number of dense granules in the patients’ platelets and, patients 1 and 3, also have some giant and lysosomal-like alpha granules (Table 1 and Fig. 1A). Some platelets almost have no granules in these patients. Staining of platelets spread on fibrinogen for the lysosomal marker CD63 showed a more centralized and fused localization of the platelets granules in the NPC1 patients P2 and P3 compared with control platelets (Fig. 1B and C). CD63 is a typical marker for platelet dense and some type of alpha granules (19). Hematological counts showed platelet numbers and mean platelet volume (MPV) at the lower normal limits (Table 1). Patients had normal counts for all types of white blood cells, reticulocytes and erythrocytes, but the mean corpuscular volume (MCV) was always low. Immunoblot analysis of NPC1 expression in platelets was reduced in the patients (Fig. 1D). The platelet granule secretion defect in our NPC1 patients only presented with a subclinical platelet defect as these patients did not yet present with any spontaneous or trauma-related bleeding problems yet.

NPC1 expression in human (erythro)megakaryocytic cells and platelets

Platelets with ultrastructural abnormalities that lead to granule and secretion defects in combination with lower platelet counts are often a result of a defective megakaryopoiesis. Therefore, we studied the expression of NPC1 in human MK cell lines (MEG01 and CHRF), in human erythromyeloblastoid cells (K562) and in in vitro differentiated MKs from CD34+ human HSCs versus normal platelets using immunoblot analysis (Fig. 2A). NPC1 expression was showed for all cell lines and primary MKs though with a different intensity of the higher (glycosylated form) and lower molecular weights for NPC1 (190 and 170 kDa) (20).

HSC differentiation studies in NPC1 patients

Since it was not possible to obtain bone marrow from these patients to study the role of NPC1 in megakaryopoiesis and platelet production, we isolated HSCs from the peripheral blood of patients P2 and P3 to compare their in vitro differentiation into MKs and erythroblasts (E) with a control subject. The differentiation experiments were performed in duplicate starting each from different amounts of HSCs (Fig. 2B). The patients had increased colony numbers of differentiated MKs at days 6 and 10 (Fig. 2B). There were no differences in colony numbers between patients and control during erythroid differentiation at day 6, while the counts at a later stage were not possible because the cells were overgrown. The MK colonies at day 10 were also larger and contained more single cells for the patients compared with the control (Fig. 2C). However, the MK cultures for the control typically showed proplatelet-forming MKs that were not observed in the cultures from P2 and P3. A hyperproliferation of only immature MKs but almost no mature proplatelet-forming MKs is typically seen in patients and mice with a genetic defect that causes thrombocytopenia with granule defects such as GATA1 deficiency (21,22). To further confirm that all colonies are MKs, a staining with an anti-GPIIa antibody was performed on these colonies. These staining showed again a hyperproliferation of small immature MKs for P2 and P3, while the control condition clearly showed proplatelet-forming MKs (Fig. 3A). The duplicate MK cultures were stained with CD63 that typically reacts with the dense and some alpha granules in mature MKs but also with the granule precursor, the multivesicular bodies (MVBs) in immature MKs (19). Figure 3B showed a different kind of granular pattern that could be due to the dense/alpha granule staining in control MKs versus MVBs staining in MKs from P2 and P3. Finally, filipin staining to reveal the cholesterol distribution in MKs was performed and showed some typical accumulation in MKs derived from the patients but in the control condition (Fig. 3C). We decided to confirm the effect of NPC1 on MK differentiation further using our available transgenic zebrafish models with fluorescently labeled blood cells (21,23) via morpholino (MO)-induced NPC1 depletion.

NPC1 is highly expressed in the developing brain, eyes and yolk syncytial layer of zebrafish embryos

To identify zebrafish NPC1, the human NPC1 amino acid sequence was used in a TBLASTP search and a 1278-amino acid protein was identified with 68% identity to the human NPC1 protein that corresponds to a gene with accession number NM_001243875.1. This sequence was later found to be identical to the NPC1 gene used by others to characterize the role of NPC1 in early embryonic zebrafish development (24). RT–PCR analysis confirmed NPC1 expression during different stages of zebrafish embryonic development (Fig. 4A). Whole-mount in situ hybridization (WISH) was used to determine the spatiotemporal expression pattern and NPC1 was detected at 24 hpf in developing brain and eyes but with the highest expression in the yolk syncytial layer (YSL) (Fig. 4B).

NPC1 depletion results in increased cell death in the brain and abnormal cellular accumulation of cholesterol

Schwend et al. (24) found that NPC1 depletion did not disrupt early cell fate or survival, but early morphogenetic movements
were delayed and increased cell death was detected. We used another NPC1 MO but showed that our ATG blocking MO completely inhibits NPC1 protein expression even at low dose of MO (200 μM) (Fig. 4C). In addition, we also detected embryos at 24 hfp with malformed heads and dysmorphic brain and eyes with increased levels of apoptosis in these regions, in a MO dose-dependent manner (Fig. 4D). Filipin staining clearly showed an accumulation of free sterols at

**Figure 1.** Platelets from NPC1 patients. (A) Electron microscopy of platelets from NPC1 patients shows platelets with a paucity of granules (arrows), absent dense granules, lysosomal-like (black arrowheads) and giant (white arrowhead) alpha granules. (B) CD63 immunostaining in spread platelets shows a granular pattern, but granules are more centralized and fused for the NPC1 patients (P2 and P3) compared with control platelets (C1 and C2). (C) Quantification of the overall coverage and the mean particle size for the CD63 staining in platelets from controls and patients. Bars represent the mean and standard deviations. *P*-value of 0.0006 by Student’s *t*-test. (D) Immunoblot analysis showing NPC1 expression in platelets from three control subjects and three NPC1 patients. Beta tubulin is the loading control.
the plasma membrane of all cells (Fig. 4E), hereby confirming that NPC1 depletion in zebrafish also results in a defective intracellular processing of cholesterol.

NPC1 depletion leads to thrombocytopenia and mild anemia

To study the role of NPC1 in megakaryopoiesis, CD41 transgenic zebrafish with GFP-labeled thrombocytes were used. Injected CD41 embryos were analyzed by life-screening for GFP<sup>high</sup> and GFP<sup>low</sup>-labeled thrombocytes in the caudal hematopoietic tissue (CHT) region at 72 hpf (Fig. 5A). According to the severity of the phenotype, embryos were divided into three sub-classes: a severe phenotype with no thrombocytes, a mild phenotype with only few (<i>n</i> < 10) thrombocytes and a normal phenotype with many non-circulating thrombocytes in the CHT region and some circulating thrombocytes. Even at a low dose of MO (200 μM), 82% of the screened embryos showed an almost complete absence of thrombocytes at 3 days post-fertilization (dpf). At a higher dose of MO (400 and 800 μM), more than 90% of the embryos had severe thrombocytopenia and all these embryos died at 5 dpf (Fig. 5A). In addition, immunoblot analysis showed reduced GFP levels in pooled samples (<i>n</i> = 30 embryos/condition) from NPC1-depleted embryos for 200 and 400 μM NPC1 MO compared with Crl MO-injected embryos (Fig. 5B). Further quantification of thrombocytes was determined by flow cytometry, measuring a total of 900 versus 183 GFP<sup>high</sup> and GFP<sup>low</sup>-labeled thrombocytes in Crl MO versus NPC1 MO-injected embryos, respectively (Fig. 5C). Especially, the GFP<sup>high</sup>-labeled thrombocytes were absent in the NPC1 condition. Interestingly, FSC plots also showed differences in thrombocyte size distribution. Since our NPC1 patients not only had lower normal limits of platelet count and MPV but also relatively low values for MCV (Table 1) and because anemia was described in NPC1-deficient mice in a C57BL/6J background (11), we also evaluated the NPC1 depletion using GATA1 transgenic fish for which erythrocytes are DSred labeled. Flow cytometric analysis of Crl MO versus NPC1 MO (200 μM) injected embryos showed a total of 5767 versus 3022 DSred-labeled erythrocytes (Fig. 5C). Again, the FSC plot of the DSred-positive erythrocytes was slightly different between the two groups.

Real-time RT–PCR confirmed a 68% reduction in relative expression of GATA1 after NPC1 depletion compared with controls (Fig. 5D). Relative expression of CD41 was also measured by real-time PCR, but CD41 levels appeared to be too low for detection after NPC1 depletion. It is assumed that the GFP<sup>high</sup> labeled (CD41 detection by RT–PCR) represent the mature thrombocytes, while the GFP<sup>low</sup> population...
includes thrombocyte precursors and stem cells (25). We detected a strongly reduced but not absent level of GFP by immunoblot analysis (Fig. 5B) and mainly absent population of GFP\textsuperscript{high} labeled thrombocytes by flow cytometry after NPC1 deletion. This would mean that the remaining population of GFP\textsuperscript{low} labeled thrombocyte precursors and stem cells are only partially affected by NPC1 depletion and represent the population that is still detected by GFP immunoblot analysis. While the GFP\textsuperscript{high} labeled thrombocytes were not detected after NPC1 depletion (CD41 RT–PCR negative), GFP\textsuperscript{low} labeled precursors and stem cells are also reduced (by 74%) but not absent as screened by RT–PCR for Scl (Fig. 5D). From these results, we concluded that the development of the complete myeloid lineage is affected by NPC1 depletion.

**DISCUSSION**

We have studied a patient (P1) with a prolonged Ivy bleeding time of 10 min and showed abnormal platelet morphology and functional aggregation and secretion tests before she was diagnosed with the NPC1 disease. Literature evidence for a role of NPC1 in hemostasis and/or hematopoiesis is very limited. Although some evidence was present for a regulatory role of NPC1 in platelet function (6,10) and platelet and red blood cell formation (6,11), this definitely required more studies. We therefore studied platelet count, function and morphology in three unrelated Niemann–Pick patients with a proven genetic and biochemical NPC1 defect. It was clear from these studies that the NPC1 defect inhibits normal platelet secretion and subsequent stimulation as shown by an absent response in the epinephrine-induced aggregation, typically present in patients with dense granule platelet abnormalities (18). Abnormalities in granule number and/or content usually originate from a defective granulopoiesis in the MK as for example found for GATA1 deficiency (21,22) or the Gray platelet syndrome (26–28). Since it was not possible to study megakaryopoiesis using bone marrow from NPC1 patients, we studied \textit{in vitro} MK and erythroblast differentiation experiments starting from peripheral blood-derived HSCs from patients and an NPC1-depleted zebrafish model.
During the HSC differentiation experiments, we could not detect an early effect on the erythropoiesis, but NPC1 deficiency has an effect on the differentiation of MKs. Patients show a hyperproliferation of early MKs that do not seem to form the terminally differentiated proplatelet-forming MKs. We and others have described a similar megakaryopoiesis defect in thrombocytopenia patients with GATA1 defects and GATA1 knockout mice (21,22) that also present with a defective granulopoiesis and associated platelet granule secretion defect. Therefore, these results together with the electron microscopy data are strongly suggestive of a platelet formation defect that causes a secondary functional defect (mainly at the levels of the granules) in NPC1 disease.

Figure 4. NPC1 expression in zebrafish and phenotype analysis of NPC1-depleted embryos. (A) RT–PCR showing NPC1 expression during different developmental stages of zebrafish embryos (30 embryos pooled for each stage). (B) WISH (30 embryos analyzed for each condition; WISH experiment performed in duplicate) in zebrafish shows NPC1 expression in the head, brain and eye at 24 hpf, using a zebrafish-specific antisense probe (left upper panel; arrow) when compared with the sense probe, used as a negative control (right upper panel). In the lower panels, a magnification of the tail region is shown to indicate the NPC1 expression in the YSL, with the specific zebrafish antisense probe (left lower panel; arrowhead) but not using a sense probe (right lower panel). (C) Immunoblot analysis for NPC1 in control (Crl) and NPC1 MO injected (200 μM) embryos (50 μg loaded from 30 deyolked, pooled and lysed embryos per condition). (D) Head phenotype analysis after NPC1 depletion using NPC1 MO at a concentration of 800, 400 and 200 μM and Crl MO, used as a negative control. A total of 100 embryos (pooled from three independent experiments) were live screened and divided according to the severity of the phenotype as indicated. Severe phenotype comprises embryos with severely dysmorphic brain and eyes and a high level of apoptosis, clearly visible as a large amount of grey cells in this region. Mild phenotype comprises embryos with brain ventricles that are still visible but showing serious malformation with apoptosis in the head and eyes. (E) Multiphoton images of filipin staining in control and NPC1-depleted embryos. Filipin staining in control embryos (left panel) shows equal distribution of cholesterol at the plasma membrane, whereas in NPC1-depleted embryos (right panel) an accumulation of free sterols at the plasma membrane as well as in the cells is visible (experiment performed in duplicate for 30 embryos).
We used CD41 transgenic zebrafish with GFP-labeled thrombocytes to show that the thrombocytopenia, obviously observed in some NPC1 patients (5), is a direct consequence of the genetic NPC1 defect and not caused by an increased destruction of platelets by their enlarged spleen and/or liver. MOs are only active during the first 4–7 days after injection,

Figure 5. Severe thrombocytopenia and anemia in NPC1-depleted embryos. (A) Thrombocyte phenotype after NPC1 depletion using NPC1 MO at concentrations of 800, 400 and 200 μM versus a Crl MO as negative control. A total of 100 embryos (pooled from three independent experiments) were live screened and divided according to the severity of the phenotype: severe (green), mild (red) or normal (blue) phenotypes. Stereomicroscope images of the CHT region at 72 hpf to visualize GFP-labeled thrombocytes (×20 original magnification) to represent each phenotype are also shown. (B) Immunoblot analysis of GFP expression for CD41 transgenic embryos (50 μg loaded from 30 deyolked, pooled and lysed embryos per condition, experiment was performed in duplicate with similar findings). NPC1 MO was injected at the indicated concentrations (μM). (C) Flow cytometric analysis of transgenic CD41+ (upper panels) or GATA1 (lower panels) embryos injected with 200 μM of NPC1 MO or Crl MO. At 72 hpf, 30 embryos for each condition were pooled and the number of GFP-positive cells was determined for a total of 500,000 total events (upper panels) or the number of DSred-positive cells was determined for a total of 100,000 total events (lower panels). These experiments were each performed in duplicate with similar findings. (D) Real-time RT–PCR using Syber Green I fluorescence and specific zebrafish primer sets for Scl, GATA1 and CD41. All relative expression levels were normalized to the endogenous reference control elfa. Graph shows the relative expression level of each marker for NPC1-depleted embryos (200 μM MO) compared with Crl MO-injected embryos. Complete experiment was performed in triplicate.
which is synchronous with the time period of thrombocyte formation (usually clearly visible in the CHT region at 3 dpf), while the liver in zebrafish is not yet functional within this time frame (29). The exact stage of zebrafish spleen formation is not known, but since it is part of the digestive tract, fully functional only at 4 dpf (30), it is unlikely that a platelet destruction would be the origin of the thrombocytopenia, already observed at 3 dpf. This would indicate that NPC1 has a major regulatory role in thrombocyte formation. We did observe severe thrombocytopenia in NPC1-depleted embryos, while the thrombocytopenia is typically mild in fetal onset NPC1 patients (5). Platelet counts were even just within normal limits in our NPC1 patients. This difference can be explained by the fact that a complete knockout in zebrafish is different from having autosomal recessive NPC1 mutations. In addition, we of course studied fetal and not adult hematopoiesis in zebrafish. HSCs in zebrafish migrate to and seed the CHT at 48 hpf, which acts as a transient hematopoietic site that gives rise to erythroid, myeloid and thromboid cells (31). The CHT is equivalent to mouse fetal liver or placenta. HSCs from the aorta-gonad-mesonephros region colonize kidney only around 48 hpf and this kidney marrow is functionally similar to mammalian bone marrow in that it gives rise to all blood lineages for the larval and adult zebrafish. NPC1 could therefore have a different role in fetal versus adult hematopoiesis.

Since NPC1 depletion in zebrafish has a role in fetal thrombopoiesis, it is expected that especially the lipid mobilization during early stages of development is important for inducing a thrombocytopenia phenotype. During the first week of larval development, the yolk contains the main supply of lipids and other nutrients needed for larval development. At the surface of the yolk, a syncytiurn, named the YSL, demarcates the extra-embryonic yolk and plays a crucial role in embryonic patterning and morphogenesis, besides its function in the transfer of nutrients to the embryo (32). We and others (24) showed that zebrafish NPC1 is highly expressed in this important lipid transfer region. Interestingly, this YSL region is in very close proximity to the CHT region for fetal hematopoiesis, but the link between defective lipid transfer and thrombocyte formation deserves further detailed studies. It is only known that disturbed cholesterol levels affect bone marrow cell mobilization (33), which indeed could result in changes of blood lineage development. Interestingly, the NPC1 depletion in zebrafish was not thrombocyte specific as we also found abnormalities in GATA1 and Scl expressing erythrocytes and stem cells, respectively. But also in some fetal onset NPC1 patients, anemia and white blood cell abnormalities were described (5). Our patients had normal erythrocyte and white blood cell counts but have borderline low MCV and at some occasions, lower hemoglobin levels.

Several publications recently raised the possibility that not cholesterol but disturbed sphingosine storage is the primary cause of the NPC1 disease. Sphingosine storage in the acidic compartment would lead to calcium depletion in these organelles, which then also results in cholesterol, sphingomyelin and glycosphingolipid storage (34). They also claim that this calcium imbalance is a new target for therapeutic intervention, since treatment of NPC1-deficient mice with curcumin, a SERCA antagonist, increased cytosolic calcium, thereby normalizing sphingolipid and cholesterol levels. We treated NPC1-depleted zebrafish with 5 and 7 μM curcumin in order to try to rescue the thrombocytopenic phenotype, but we did not observe any correction (data not shown). However, the pharmacokinetics of curcumin in zebrafish were recently shown to be different compared from those of rats and even to cause developmental defects including shorter body axis with hooked tails, spinal column curving, edema in pericardial sac and retarded yolk sac resorption (35). The question whether disturbed cholesterol levels or imbalanced calcium with sphingosine storage plays a dominant role in the NPC1-depleted zebrafish phenotypes remains open.

In conclusion, this study shows morphological and functional platelet abnormalities in three unrelated NPC1 patients, more specifically, platelets with granulopoiesis defects typically arising during megakaryopoiesis. In addition, NPC1 depletion in zebrafish further supports an important role for NPC1 in thrombocyte formation. We show that NPC1 depletion does not only affect megakaryopoiesis but also leads to anemia and a general defect in stem cell development; however, further studies are needed to link these phenotypes to a defective lipid storage metabolism.

MATERIALS AND METHODS

Patient study

Informed consent was obtained from all participants and/or their legal representatives. The Ethics Committee of the University of Leuven approved the study.

Hematological counts and functional platelet studies

EDTA anticoagulated blood was analyzed on an automated cell counter (Cell-Dyn 1300; Abbott Laboratories, Abbott Park, IL, USA) to determine blood cell counts. Platelet-rich plasma (PRP) was prepared by centrifugation (15 min at 150 g) of whole blood anticoagulated with 3.8% trisodium citrate (9:1). The PRP was used for functional platelet studies and electron microscopy, as described previously (18). In short, aggregation studies were carried out by adding Horm collagen, epinephrine or ADP at the indicated concentrations. ATP secretion tests were performed after stimulation of platelets with Horm collagen 2 μg/ml and ADP 10 μM. Flow cytometry to measure expression of CD61, CD41, CD42a, CD42b and CD62P was performed as described previously (36).

Platelet spreading and staining

Platelet spreading and staining was performed as described (37). Platelets were incubated with a 1:50 anti-CD63 (BD Biosciences PharMingen, San Diego, CA, USA) of anti-GPIIIa antibody overnight at 4°C. After five washing steps with PBS, cells were incubated with a specific secondary antibody diluted 1:200 (Alexa Fluor; Life technologies) together with phalloidin rhodamine (Sigma, Poole, UK) for F-actin staining during 45 min at 37°C. Analysis was done using a Zeiss Axiovert 100M confocal microscope (Carl Zeiss Inc., Gottingen, Germany). Fluorescent intensities were quantified using the
Cell cultures and differentiation of CD34+ HSCs

Human megakaryocytic cell lines MEG01 and CHRF and human erythroid-myeloblastoid cells K562 were grown as described previously (38,39). Human CD34+ cells were isolated from peripheral blood by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Human CD34+ cells (0.5 and 1 × 10^4 and 0.5 and 1 × 10^3, respectively) were cultured in duplicate in Megacult-C 04973 and Methocult 04964, according to the manufacturer’s instructions (Stem Cell Technologies). The total number of MK and erythroblasts colonies were counted 6 and 10 days later using a light microscope (Leica DM RBE, Wetzlar, Germany) in cultures performed in duplicate for each start condition of HSCs. Slides were processed for stained as described by the manufacturer (Stem Cells). Stainings of MK cultures were done with the monoclonal mouse anti-GPIIIa antibody and 50 μg/ml filipin (Sigma Chemical Co., St Louis, MO, USA) or with anti-CD63 and photographed at 40×/0.5 magnification with a Zeiss Axiocam MRc camera using AxioVision software.

MKs and erythroblasts colony assays and stainings

Human CD34+ cells were isolated from peripheral blood by magnetic cell sorting (Miltenyi Biotec, Auburn, CA, USA) and were cultured in the StemSpan SFEM medium (Stem Cell Technologies, Vancouver, Canada), supplemented with 20 ng/ml TPO, 10 ng/ml SCF, 10 ng/ml IL-6 and 10 ng/ml FLT-3 (Peprotech, London, UK) as described previously (38).

MO injection in zebrafish

Tg(cd41:EGF) (25) and Tg(gata1:dsred) (40) zebrafish embryos were injected at the 1-cell stage with a start codon MO ATG MO (5’-TGTTTTGCTCCCGAGCAGAGCAT-3’) at the indicated concentrations (200–800 μM). Off-target effects were assessed by including a standard control MO or buffer-injected embryos. MOs were designed by Gene-Tools LLC (Philomath, OR, USA). Embryos were life-screened at 1, 2 and 3 dpf using a Zeiss Lumar V12 and images were captured with a Zeiss Axiovision.

Conflict of Interest statement. None declared.

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