Kindlin-3 in hematopoietic integrin activation: Absence in leukocyte adhesion deficiency type III
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CHAPTER 3

Leukocyte Adhesion Deficiencies

Edith van de Vijver, Timo K. van den Berg, Taco W. Kuijpers

During inflammation, leukocytes play a key role in maintaining tissue homeostasis by elimination of pathogens and removal of damaged tissue. Leukocytes migrate to the site of inflammation by crawling over and through the blood vessel wall, into the tissue. Leukocyte adhesion deficiencies (i.e., LAD-I, -II, and LAD-I/variant, the latter also known as LAD-III) are caused by defects in the adhesion of leukocytes to the blood vessel wall, due to mutations in the genes encoding β2 integrin (ITGB2), a GDP-fucose transport protein (SLC35C1) and kindlin-3 (FERMT3), respectively. Patients experience recurrent nonpussing bacterial infections and neutrophilia, often preceded by delayed separation of the umbilical cord, and additional symptoms depending on the subtype. For LAD-I and LAD-III, the only curative treatment is hematopoietic stem cell transplantation. In case of LAD-II, oral fucose supplementation may invert the immune defect, but additional mental retardation is hardly improved.
INTRODUCTION

**Leukocyte recruitment and extravasation**

During inflammation, circulating leukocytes migrate to the site of infection following a gradient of chemotaxins in a process called chemotaxis. Chemotaxins may be derived either from the infected tissue or local complement activation, or directly from the pathogens themselves, and diffuse within the tissue into the local vasculature. These gradients of chemotaxins recruit the leukocytes in interplay with factors expressed locally on the luminal side of blood vessel endothelial cells. Neutrophils are short-living leukocytes that are recruited early in the inflammatory response.

Leukocytes following the chemotaxon gradient towards the site of infection have to leave the blood stream, in a process called extravasation (Figure 1). Extravasation is a multi-step process involving adhesion molecules, in which chemotaxins function as activating agents or (pro-)inflammatory mediators. The first step of extravasation consists of initial contact between endothelial cells and leukocytes marginated by the fluid flow of the blood. L-selectin (CD62L) on leukocytes plays a role herein, contacting several cell adhesion molecules on endothelial cells. Within the local environment of an inflammatory tissue reaction, the endothelium begins to express the adhesion molecules P-selectin (CD62P) and later on E-selectin (CD62E). The low-avidity interaction of these selectins with their fucosylated ligands on the opposite cells forces the leukocytes to slow down and start a rolling movement along the vessel wall (Figure 1).

In contrast to the low-avidity binding of leukocytes to selectins, the final step of firm adhesion and subsequent migration depends on stable interaction between integrins on the leukocytes and their ligands on the endothelial cells. Integrins are type I transmembrane glycoproteins that

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**Figure 1. Leukocyte recruitment and extravasation.** Leukocytes migrate to the site of inflammation following a gradient of chemotaxins. The cells slow down due to transient interactions between selectins and their glycosylated ligands, which are defective in LAD-II. Next, stable adhesion by leukocyte integrins, absent in LAD-I, to ligands on the endothelium results in leukocyte arrest. Activation of blood cell integrins is decreased in LAD-III. Healthy neutrophils extravasate subsequent to firm adhesion.
form heterodimers via non-covalent association of their α and β subunits, with sizes of 120-170 kDa and 90-130 kDa, respectively. In mammals, 18 α and 8 β subunits form 24 known combinations, each of which can bind to a specific repertoire of cell-surface, extracellular matrix or soluble protein ligands. The β2 integrin receptor subfamily is selectively expressed on leukocytes and comprises four different heterodimeric proteins, each of which contains a different α subunit: i.e. αLβ2 (LFA-1; CD11a/CD18), αMβ2 (CR3; CD11b/CD18), αXβ2 (gp150,95; CD11c/CD18); and αDβ2 (CD11d/CD18), the latter only being expressed on macrophages. The β2 integrins bind to adhesion molecules on endothelial cells (intercellular adhesion molecule [ICAM]-1 and ICAM-2), and to several complement factors. The main β2 integrin on neutrophils is CR3.

As mentioned above, slowly rolling leukocytes are able to recognize concentration differences in a gradient of chemotaxins and to direct their movement towards the source of these agents. Although the details of this process remain unknown, the gradient most likely causes a difference in the number of ligand-bound chemotaxin receptors on either side of the cell, thereby inducing the cytoskeletal rearrangements needed for movement. Since adhesion molecules such as the β2 integrins are essential for the connections with the tissue cells or with the extracellular matrix proteins, these connections must be formed at the front of the moving leukocytes and broken at the rear end. Moreover, for continued sensing of the chemotaxin gradient, the chemotaxins must dissociate from their respective receptors for repeated usage. This occurs through internalization of the ligand-receptor complex, intracellular disruption of the connection, and transport of the free receptor to the front of the cell, followed by reappearance of the free receptor on the leukocyte surface. Within the infected tissue, the chemotaxin gradient persists and leukocyte migration is maintained.

**Integrin activation**

The ligand specificity of integrins is determined by their large extracellular ligand-binding head domain, which is composed of several domains of both the α and β subunit. The head domain is attached to the membrane via two flexible legs (one from each subunit), which terminate intracellularly as short cytoplasmic tails. This domain architecture of integrins underlies their ability to transduce bidirectional signals across the plasma membrane: “inside-out” and “outside-in” (Figure 2). Leukocyte activation, e.g. as a result of chemokine binding to chemokine receptors, ligand binding to selectins, or antigen binding to the T-cell receptor, and subsequent intracellular signaling induces conformational changes in the extracellular regions of the β2 integrins, leading to an enhanced affinity for their ligands (“inside-out” signaling). In addition, integrins cluster in larger complexes, which increases their ligand avidity. Binding to extracellular ligands leads to further conformational changes of the β2 integrins, resulting in high ligand affinity and subsequent recruitment of cytosolic proteins and the initiation of downstream signaling cascades that regulate cell spreading and alter gene expression, cell proliferation, differentiation and apoptosis (“outside-in” signaling).

The common activator of most, if not all, integrins is talin, a large cytoskeletal protein that acts as an allosteric activator of integrins by inducing their ligand-binding affinity. The head domain of talin contains a FERM (4.1 protein, ezrin, radixin, moesin) domain, consisting of three subdomains, F1, F2 and F3. The latter, the F3 subdomain, contains a phosphotyrosine-binding (PTB)-like domain that binds to the NPxY/F motif found in the membrane-proximal cytoplasmic
region of several β integrins. The head domain is connected to a long cytoplasmic rod which can interact with the cytoskeleton, allowing talin to contribute substantially to adhesion and motility via “outside-in” signaling cascades.

While binding of talin appears to be the final step in integrin activation, the activity of talin itself may be regulated by a variety of cell-type specific signaling pathways. In a study on CHO cells expressing platelet integrins αIIb and β3, it was proposed that PKCa, activated by phorbol-12-myristate-acetate (PMA) or T cell receptor stimulation, phosphorylates PKD1, which associates with a small GTPase from the Rap or Ras family that in turn activates the integrin upon formation of an activation complex with its effector protein Rap1-GTP-interacting adaptor molecule (RIAM) and talin.

More recently, kindlin proteins have arisen as key players in integrin activation. Kindlins comprise a family of integrin-binding proteins. In man, the family consists of three members – kindlin-1, -2 and -3 – that share a high degree of homology. Kindlin-3 is expressed in all hematopoietic cell types, where it plays an important role in a variety of functions depending on integrin-mediated adhesion, such as platelet clot formation and leukocyte extravasation.20,21 Biochemical studies have confirmed that all kindlins directly bind synthetically generated cytoplasmic tails of β1, β2 and β3 integrins. Since kindlins possess a FERM domain that is homologous to that of talin, it was hypothesized that kindlins, like talin, interact with the

**Figure 2. Leukocyte integrin activation.** Integrins are heterodimers of an α and a β chain, consisting of an extracellular ligand-binding head, a transmembrane domain and two short cytoplasmic tails. Leukocyte activation, e.g. by chemotaxin binding, causes “inside-out” signaling. This intracellular signaling – in which talin and kindlin play an important role - induces a conformational change resulting in increased ligand affinity. Subsequent ligand binding initiates downstream “outside-in” signaling, where talin and kindlin may participate again, which leads to reversible shuttling to a high affinity conformation. Together with integrin clustering, this high affinity conformation allows adhesion mediated processes such as cell spreading and chemotaxis.
membrane-proximal NPxY/F motif in the cytoplasmic tail of β integrins. However, recent studies have demonstrated that the kindlin-binding site of β integrins is distinct from the talin-binding site, i.e. at a membrane-distal NxxY/F motif in the cytoplasmic integrin tail. Biochemical studies with mutants of kindlin-2 have shown that the PTB domain in F3 is, in analogy to talin, essential for integrin binding, in addition to a requirement of the N-terminus of the protein for interaction with β3. Kindlins are essential for “inside-out” integrin activation and recent reports point to an additional role in downstream “outside-in” cascades.

**LAD**

Leukocyte adhesion deficiencies (i.e., LAD-I, -II and -III, the latter also known as LAD-1/variant) are immunodeficiencies caused by defects in the adhesion of leukocytes (especially neutrophils) to the blood vessel wall. As a result, patients with any LAD subtype suffer from severe bacterial infections and neutrophilia, often preceded by delayed separation of the umbilical cord (Table 1). LAD-II is characterized by additional developmental problems, whereas in LAD-III, the immune defects are supplemented with a Glanzmann Thrombasthenia-like bleeding tendency.

### LEUKOCYTE ADHESION DEFICIENCY TYPE I

Leukocyte adhesion deficiency type I (LAD-I) is an autosomal recessive disorder caused by decreased expression or functioning of CD18, the β subunit of the leukocyte β2 integrins. LAD-I was first described in the early 80s and since then several hundreds of patients have been reported. Mutations are found in ITGB2 (integrin β, CD18), the gene located at 21q22.3 (OMIM *600065) that encodes the β2 integrin. So far, 86 different mutations have been reported. Usually, this leads to the absence or decreased expression of the β2 integrins on the leukocyte surface, but sometimes a normal expression of nonfunctional β2 integrins is found. Decreased expression of the common β2 subunit leads to a similar decrease in the expression of all four a subunits on the leukocyte surface.

**Physical Examination**

LAD-I manifests by recurrent, life-threatening bacterial and fungal infections, primarily localized to skin and mucosal surfaces. Infections are usually apparent from birth onward, together with severe septicaemia in some patients, and a common presenting feature is omphalitis with delayed separation of the umbilical cord (Figure 3). Later on patients develop non-purulent, necrotizing infections of the skin and mucous membranes, resulting in a high mortality rate at early age. Absence of pus formation at the sites of infection is a hallmark and the infections have a high tendency for recurrence; secondary bacteremias may also occur. Among patients who survive infancy, severe gingivitis and chronic periodontitis are major features. Fungal infections may present in individual cases.

**Imaging and Additional Testing**

LAD-I patients exhibit mild to moderate leukocytosis, especially granulocytosis, with neutrophil counts reaching levels above 100,000/ml during acute infection. Due to the lack of adhesive
Table 1. Characteristics of LAD-I, -II and -III

<table>
<thead>
<tr>
<th>Physical examination</th>
<th>LAD-I</th>
<th>LAD-II</th>
<th>LAD-III</th>
</tr>
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<tbody>
<tr>
<td>Recurrent non-purulent bacterial and fungal infections</td>
<td>++ (skin &amp; mucosal, gingivitis, periodontitis; necrotizing)</td>
<td>+ (decreasing during childhood; periodontitis)</td>
<td>+ (all have bacterial, some have fungal)</td>
</tr>
<tr>
<td>Delayed separation of umbilical cord, wound healing defect</td>
<td>+ (common omphalitis)</td>
<td>-</td>
<td>+ (some omphalitis)</td>
</tr>
<tr>
<td>Dysmorphism</td>
<td>-</td>
<td>+ (growth retardation, short stature, coarse face)</td>
<td>-</td>
</tr>
<tr>
<td>Neural abnormalities</td>
<td>-</td>
<td>+ (mental retardation, autism, convulsions, cerebral atrophy)</td>
<td>-</td>
</tr>
<tr>
<td>Bleeding tendency</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<table>
<thead>
<tr>
<th>Imaging and Additional Testing</th>
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<tbody>
<tr>
<td>Genetic defect</td>
<td>+ (ITGB2)</td>
</tr>
<tr>
<td>Leukocytosis</td>
<td>+ (20.10^6 up to 140.10^6/ml)</td>
</tr>
<tr>
<td>Erythrocytosis</td>
<td>-</td>
</tr>
<tr>
<td>Thrombocytosis</td>
<td>-</td>
</tr>
<tr>
<td>Expression of integrins (CD18, CD11b)</td>
<td>1-30%, or present but dysfunctional</td>
</tr>
<tr>
<td>Expression of fucosylated glycoconjugates</td>
<td>+</td>
</tr>
<tr>
<td>Neutrophil defects</td>
<td>+ (firm adhesion, chemotaxis)</td>
</tr>
<tr>
<td>Platelet defects</td>
<td>-</td>
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</tbody>
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<table>
<thead>
<tr>
<th>Therapeutic Options</th>
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<tbody>
<tr>
<td>Antibiotics</td>
<td>+</td>
</tr>
<tr>
<td>HSCT</td>
<td>+ (severe vs moderate)</td>
</tr>
<tr>
<td>Fucose supplementation</td>
<td>-</td>
</tr>
<tr>
<td>Granulocyte transfusion</td>
<td>+ (potential benefit established)</td>
</tr>
<tr>
<td>Thrombocyte transfusion</td>
<td>-</td>
</tr>
<tr>
<td>Survival without HSCT</td>
<td>Severe form: low; moderate form: better</td>
</tr>
</tbody>
</table>

Abbreviations: Avg, average; Hb, hemoglobin; HSCT, hematopoietic stem cell transplantation; Plt, platelets; sLeX, sialyl Lewis X; LeX, Lewis X.

capacity only few, if any, leukocytes are present at the sites of infection, which are most often caused by *Staphylococcus aureus*, Gram-negative enteric organisms or fungi.

Definitive diagnosis of LAD-I is based on genetic analysis, revealing mutations in *ITGB2*. Flow cytometry with antibodies to detect CD18 allows discrimination of two forms of LAD-I,
LEUKOCYTE ADHESION DEFICIENCIES

i.e. a severe form with less than 2% CD18 expression and a moderate form with 2-30%. A rare third group with severe symptoms exhibits normal expression of a functionally-defective mutant protein. The severity of clinical presentation and complications in LAD-I correlate with the percentage of leukocytes demonstrating normal CR3 cell surface expression and/or degree of molecule deficiency. Patients with severe LAD-I exhibit earlier, more frequent, and more serious episodes of infection, often leading to death in infancy, whereas patients with a moderate to mild phenotype experience fewer serious infectious episodes and commonly survive into adulthood.

Extensive in vitro studies on neutrophil functions have demonstrated a marked defect in random migration as well as chemotaxis to various chemoattractants. Adhesion to and transmigration across endothelial cell layers were found to be severely impaired. Neutrophils fail to mobilize to skin sites in the in vivo Rebuck skin-window test.

**Therapeutic Options**
Antibiotics are commonly used to prevent and specifically treat acute or recurrent infections, and patients affected with the moderate form may survive to adulthood solely with antibiotics. As a curative treatment, hematopoietic stem cell transplantation (HSCT) is the only approach, and is most often the treatment of choice for patients suffering from the severe form of LAD-I.

Both reduced-intensity and myeloablative conditioning regimens are currently being used in HSCT of LAD-I patients. With myeloablative conditioning, more complete depletion of host marrow can be achieved, thereby decreasing the possibility of mixed chimerism and the risk of rejection. However, pre-transplant infections in immunodeficient patients lead to a high rise in mortality rate with this regimen, especially in patients suffering from comorbid complications.

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**Figure 3. Tissue necrosis after removal of the umbilical stump.** LAD-I and -III patients commonly present with delayed separation of the umbilical cord, often followed by omphalitis, and resulting in tissue necrosis in some cases.
According to studies by the group of Hamidieh et al., use of the less toxic reduced-intensity conditioning (RIC) regimen is found to be a more safe and feasible therapeutic approach in the treatment of LAD-I patients. Recipients of RIC transplant, those with either full or mixed chimerism, had a long-term survival rate with no manifestation of LAD-I symptoms.

Further, granulocyte transfusions have been reported as a successful supplementation to LAD-I treatment. A patient who was suffering for 18 months from an ecthyma gangrenosum (EG) lesion, despite treatment with targeted antibiotics and anti-fungal therapy, has been cured by massive granulocyte transfusions in association with G-CSF. Overall, the role of granulocyte transfusion in acute infectious episodes is debatable owing to its side effects.

More recently, gene therapy has arisen as a potential treatment. Although so far gene therapy was unsuccessful in human LAD-I patients, a study using foamy virus vectors in a canine leukocyte adhesion deficiency model was very promising.

**Clinical Outcomes**

The moderate form of LAD-I can often be controlled with prompt use of antibiotics during acute infectious episodes and, sometimes, prophylactic antibiotics. For patients suffering from severe LAD-I, transplantation is commonly required at an early age. A multicenter study involving 36 patients demonstrated an overall survival rate of 75%, with best results from HLA-matched stem cell donors.

**Complications and Concerns**

Frequent use of antibiotics may result in resistance of the bacteria. HSCT can be unsuccessful especially in case of an incompletely matched donor. Survival of HSCT treatment is lower than average for immunocompromised patients owing to the risk of pre-transplant infections. Although granulocyte transfusions have added to treatment in some cases, the side effects may induce additional risks.

**LEUKOCYTE ADHESION DEFICIENCY TYPE II**

The rare LAD-II syndrome was first reported in 1992 in two unrelated Arab Israeli boys, and up to date less than 10 patients have been reported, most of them from the Middle East. Patients with LAD-II (OMIM #266265) have a defect in the fucosylation of various cell surface glycoproteins, some of which function as selectin ligands, such as sialyl Lewis X carbohydrate groups (sLeX, CD15a). As a result, the initial “rolling” of leukocytes over the endothelial vessel wall in areas of inflammation, which is mediated by reversible contact between L-selectins on the leukocytes and E- or P-selectins on the endothelial cells with their respective sialated fucosyl ligands on the opposite cells, is disturbed. Without rolling, the leukocytes cannot slow down and stably adhere, and in this way LAD-II leads to decreased leukocyte extravasation and recruitment to the site of infection. Fucosylation is important as well for several unrelated functions, and LAD-II patients present as a result with additional symptoms, including mental and growth retardation.

The molecular defect in LAD-II has been identified as a deficiency in a Golgi GDP-fucose transport protein (GFTP). This protein is encoded by SLC35C1 (Solute carrier family...
35 member C1), or FUCT1 (GDP-fucose transporter 1) at 11p11.2 (OMIM *605881), in which 7 different mutations have been reported so far. Since the genetic cause reveals that the defect involves glycosylation, LAD-II has now been categorized as one of the group of the congenital disorders of glycosylation (CDG), and has been reclassified as CDG-IIc.

Physical Examination

The clinical course of LAD-II with respect to infectious complications is a milder one than LAD-I, in accordance with lower leukocyte counts. While rolling is defective in LAD-II patients, the adhesion and transmigration via β2 integrin is intact, thereby permitting apparently some neutrophil mobilization to sites of inflammation, and allowing some level of neutrophil defense in tissues. In addition, the mechanisms of β2-integrin activation are still intact. Although recurrent bacterial infections occur in almost all patients, they are often not very severe and not resulting in overt wound healing defects or necrotic lesions as in LAD-I. Most infections occur in the first years of live, although periodontitis has been reported at later age.

Figure 4. Clinical stigmata in LAD-II patients. Defective fucosylation results in growth retardation and a coarse face. Long eyelashes, a broad and depressed nasal bridge, a simian crease, and dorsally positioned second toes are the clinical stigmata of a patient with LAD-II. (From Marquardt T, Brune T, Luhn K, et al. Leukocyte adhesion deficiency II syndrome, a generalized defect in fucose metabolism. J Pediatr 1999;134(6):681–8; with permission.)
However, LAD-II patients present with other abnormal features, such as growth retardation (short stature), mental retardation and a coarse face (Figure 4).38,39 Patients are born at term, with no apparent dysmorphism, but severely impaired postnatal weight gain and microcephaly were reported for most patients. In some families intrauterine growth retardation was sufficient to screen for LAD-II prenatally. In addition, convulsions, cerebral atrophy and autistic features were reported for more than half of the patients.38 One patient had coronal craniosynostosis.42

It should be mentioned that the early and late features of LAD-II, namely, moderate immunodeficiency accompanied by neutrophilia in the first few years of life and severe mental retardation and short stature in childhood, are also prominent features of other congenital disorders of glycosylation (CDGs).

Imaging and Additional Testing

The biochemical hallmark of LAD-II is a lack of expression of fucosylated glycoconjugates, such as the Lewis antigens Lewis X (LeX) and sialyl Lewis X (sLeX) on leukocyte proteins, α1,6-core fucosylated N-glycans on fibroblast proteins and blood group antigen H on erythrocytes, the latter known as the rare Bombay blood group phenotype.38,43 Expression of CR3 on LAD-II neutrophils is normal.44,45

Neutrophil values range from 6,000 to >50,000/ml in absence of infection up to 30,000 to 150,000/ml during infectious episodes.36,46 With intravital microscopy, it was observed that LAD-II neutrophils roll poorly, only 5%, whereas the rolling fraction of control and LAD-I neutrophils is around 30%.38 The neutrophil counts remain high during childhood and then drop at adolescence; this finding might be explained by an improvement in adaptive immunity with age, providing better defense against infections and reducing the stimuli for neutrophilia.38

Final proof of LAD-II arises from genetic analysis of the \textit{SLC35C1} gene. The mutation seems to determine the severity of LAD-II: whereas GFTP is improperly located in the ER in some patients, it is directed to the Golgi but still dysfunctional in others, the latter correlated with a milder immunological phenotype.38

Therapeutic Options & Clinical Outcome

Infections are commonly treated with antibiotics. In addition, high-dose oral supplementation of fucose had strong beneficial effects in some patients.39,43 During 9 months of treatment with fucose of the first patients, infections and fever disappeared, elevated neutrophil counts returned to normal, and in one of the patients even psychomotor capabilities improved. However, fucose supplementation is not successful in all patients, since treatment of two Israeli Arab patients did not exhibit a similar beneficial response.48 In addition, for one of the treated patients treatment led to an autoimmune response against refucosylated antigens.47 Upon discontinuation of the therapy, selectin ligands were lost and neutrophil counts increased again within 7 days.49

Complications and Concerns

The metabolic pathways causing the severe psychomotor and growth retardation are still unclear. Oral fucose supplementation may cure immunologikal symptoms in some cases, but developmental delay is hardly improving.39
LEUKOCYTE ADHESION DEFICIENCY TYPE III, TYPE-1/VARIANT

LAD-III, also known as LAD-1/v, was first described in 1997 by our group and has now been identified in about 20 families worldwide. In addition to recurrent non-purulent infections, LAD-III patients exhibit a severe Glanzmann Thrombasthenia-like bleeding disorder. Families have often lost newborns within weeks after birth, previous to the diagnosis of the reported patients, demonstrating the high mortality rate of LAD-III patients. The bleeding disorder originates from a platelet defect, indicating that the signaling defect also affects the b3 integrin fibrinogen receptor aIIbb3 on blood platelets.

The molecular defect of this variant form of LAD (OMIM #612840) has since 2010 been assigned to mutations in FERMT3 (fermitin family homolog 3) at 11q13.1 (OMIM *607901). FERMT3 encodes kindlin-3, a protein involved in inside-out signaling to all blood cell-expressed β integrins (β1, β2 and β3). So far, 11 different mutations in FERMT3 have been reported. A discussion has taken place in the literature about the importance of a genetic variation in the gene encoding CalDAG-GEF1 (a guanine nucleotide exchange factor for Rap1, involved in integrin activation) in some patients with LAD-III, in addition to mutations in FERMT3 found in these patients. However, since the functional defect in such patients can only be corrected by reconstitution with kindlin-3 and not by reconstitution with CalDAG-GEF1, this variation in CalDAG-GEF1 is of no importance for the functional defect in LAD-III patients. Experiments with kindlin-3 knockout mice by Moser et al. had led to the original hypothesis of kindlin-3 deficiency in LAD-III - confirmed as indicated by the description of various mutations - and have subsequently been used as a tool to delve into LAD-III pathology.

Physical Examination

LAD-III patients suffer from severe recurrent non-purulent infections, often preceded by delayed umbilical cord detachment with or without omphalitis, and leukocytosis as seen in LAD-I. In addition, LAD-III patients are affected by a bleeding tendency, similar or more severe than exhibited by Glanzmann Thrombasthenia patients. Some patients suffering from LAD-III may also present with an osteopetrosis-like bone defect in addition to the increased bleeding tendency and recurrent infections. A prominent osteopetrotic phenotype was also observed in the kindlin-3 knockout mice. The cause of this osteopetrosis might lie within the osteoclasts, which represent macrophage-like hematopoietic cells critical for bone resorption. Bone resorption requires the formation of a so called ‘sealing zone’ that depends on αvβ3 integrin-mediated adhesion to the bone, thereby explaining the skeletal defect. However, the prevalence and manifestations of osteopetrosis differ largely among LAD-III patients, as unaffected bone formation is also found (Figure 5). The reason for this heterogeneity has remained unclear.

Imaging and Additional Testing

As for LAD-I and -II, LAD-III should be confirmed by genetic analysis, in this case revealing mutations in FERMT3. Expression of integrins on neutrophils and platelets (i.e. αIIbβ3, α1β2) is normal or slightly increased, and integrin activation can be induced by artificial stimulation with monoclonal antibodies (mAbs) or cations. Based on the persistent leukocytosis, many of the patients were suspected to suffer from juvenile myelomonocytic leukemia (JMML). However,
the increased sensitivity of bone marrow (BM) or blood cells to GM-CSF as the hallmark for JMML, is negative in LAD-III.

Many tests have been performed on LAD-III neutrophils. One example of an assay to discriminate between LAD-I and LAD-III neutrophils is the NADPH oxidase screening test with unopsonized zymosan (UZ) as described by our group. UZ is used to induce uptake and NADPH oxidase activity in purified neutrophils based on the requirement for kindlin-3-dependent CR3 activation before uptake of the zymosan. The response is absent in both types of LAD, but activation and subsequent zymosan uptake can be induced by high Mg²⁺ concentrations only in case of LAD-III, proving that CR3, once in its active conformation, is functional in these patients. Similarly, neutrophil adhesion to CR3 ligands is absent in response to several chemoattractants, but can be induced with Mn²⁺ upon artificial integrin activation.

In addition to the recurrent infections, LAD-III patients suffer from a bleeding tendency, which has been reported as more severe than in Glanzmann Thrombasthenia. With a novel flowcytometric aggregation assay, both syndromes can be discriminated based on formation of small aggregates. Platelets from Glanzmann patients are still capable of forming small aggregates upon collagen stimulation, whereas platelets from LAD-III patients are not. These aggregates require functional GPIa/IIa (integrin α2β1), thus explaining the clinically more severe bleeding manifestations in LAD-III patients, in which all platelet integrins are functionally defective.
**Figure 6. Abnormal erythrocyte phenotype.** Dacrocytes (teardrop-shaped; left 2 arrowheads) and elliptocytes (right arrowhead) characterize the erythrocyte population of LAD III, although the phenotype is less severe than in kindlin-3−/− mice.

Erythrocytes of kindlin3−/− mice are abnormally shaped, with striking protrusions and invaginations. The cells have altered expression levels of cytoskeletal proteins, and the mice are severely anemic. In human LAD-III patients, we and others have seen less prominent alterations of the erythrocyte population, including dacrocytes (tear drop-shaped) and elliptocytes (Figure 6).56 Altered levels of cytoskeletal proteins have not been reported.

Splenomegaly or hepatosplenomegaly has been observed in almost all patients, in some only later during infancy, which may result from extramedullary hematopoiesis. Besides expression in hematopoietic cells, low kindlin-3 levels have been reported in endothelial cells as well, although the biological significance is unclear.66

**Therapeutic Options**

Patients with LAD-III need prophylactic antibiotics as well as repeated blood transfusions, but the only curative therapy is HSCT. While untransplanted, the urge for transfusion differs per patient and can rise to more than 20 and 50 transfusions per year for erythrocytes and platelets, respectively.64 In addition, granulocyte transfusions have been used in at least one patient and are believed to increase pathogen clearance.64
Clinical Outcomes
The survival of untransplanted LAD-III patients is low, and the high mortality is further demonstrated by the incidence of deceased siblings who were not diagnosed but suffered from similar symptoms. Less than 4 patients have so far survived childhood without HSCT, and the oldest reported patient has reached the age of 20, although the need for platelet transfusions has increased to 1-2 transfusions per week (unpublished data). Upon successful HSCT, patients may continue live without further symptoms.

Complications and Concerns
Whereas the success rate of HSCT is improving over the last years, pre-transplant infections and the bleeding disorder often cause major complications in the treatment of LAD-III patients. In addition, osteopetrosis may complicate the conditioning regimen.67

ACKNOWLEDGEMENTS
We gratefully acknowledge Prof. Dirk Roos for critically reading the manuscript.

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