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Title: Clinical and Laboratory Findings in Patients with Leukocyte Adhesion Deficiency Type I: A Multicenter Study in Turkey

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Abbreviations

CADD: combined annotation dependent depletion

CD18: β-chain of β2 integrins

CMV: cytomegalovirus

EBV: epstein-barr virus

FERMT3: fermitin family homolog 3

gnomAD: genome aggregation database

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HLA: human leukocyte antigen

HSCT: hematopoietic stem cell transplantation

IGV: integrative genomics viewer

IL-17: interleukin-17

IL-23: interleukin-23

INDEL: insertion and deletion

ITGB2: integrin subunit beta 2

LAD-I: leukocyte adhesion deficiency type I

MAF: minor allele frequency

NGS: next-generation sequencing

PolyPhen2: polymorphism phenotyping *v2*

SIFT: sorting intolerant from tolerant

SLC35C1: solute carrier family 35 member c1

SNP: single nucleotide polymorphism

UC: umbilical cord

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding authors, IY, IT.

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Disclosure of Conflicts of Interest

The authors declare that they have no conflict of interests.

Ethical Approval

The study complied with the Declaration of Helsinki and was approved by the ethics committee of Hacettepe University.

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All participants provided written and informed consent. All co-authors give their formal written Consent to Publish prior to the submission of each version of the paper.

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Abstract

Leukocyte adhesion deficiency type I is rare primary immunodeficiency disorder characterized by mutations in the ITGB2 gene encoding CD18. We present clinical and immunological features of 15 patients with leukocyte adhesion deficiency type I (LAD-I). Targeted next-generation sequencing was performed with either a primary immunodeficiency gene panel comprising 266 genes or a small LAD-panel consisting of five genes for genetic analysis. To measure the expression level of integrins on the leukocyte surface, flow cytometry analysis was performed. The median age of the patients at diagnosis was 3 (1-48) months. Eleven (73%) of the 15 patients had LAD-I diagnosis in their first 6 months, and fourteen (93%) patients had consanguineous parents. Delayed separation of the umbilical cord was present in 80% (n=12) of the patients in our cohort, whereas omphalitis was observed in 53% (n=8) of the patients. Leukocytosis with neutrophil predominance was observed in 73% (n=11) patients. Nine distinct variants in the *ITGB2* gene in 13 of the 15 patients with LAD-I were characterized, of which two (c.305_306delAA and c.779_786dup) are novel homozygous mutations of *ITGB2*. Four unrelated patients from Syria had a novel c.305_306delAA mutation that might be a founder effect for patients of Syrian origin. Four (27%) patients underwent HSCT. Two of them died because of HSCT complications, and the other two are alive and well. Early differential diagnosis of the

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patients is critical in the management of the disease and genetic evaluation provides a basis for family studies and genetic counseling.

Key words

leukocyte adhesion deficiency type 1 (LAD-I), integrin, CD18, ITGB2, HSCT

Introduction

Leukocyte adhesion deficiency type I (LAD-I) is a rare primary immunodeficiency with a prevalence of 1/100,000 [1]. LAD-I is an autosomal recessively inherited disorder caused by mutations in *ITGB2* gene encoding the β-chain (CD18) of β2 integrins, located at chromosome 21[2,3]. CD18 expression is essential for integrin dimerization, adhesion between leukocytes and endothelial cells, and processes such as extravasation and antimicrobial activity[4,5]. Defective or deficient CD18 impairs leukocyte mobilization into inflammation sites, causing tissues to become susceptible to long-lasting bacterial infections. The IL-23/IL-17 axis also becomes dysregulated, and wound healing is diminished[6].

Clinical manifestations of LAD-I are delayed separation of the umbilical cord (UC), omphalitis and recurrent infections. Besides recurrent bacterial infections of skin and mucosal surfaces, gingivitis, periodontitis, otitis media and anemia secondary to severe infections are other symptoms[7,8]. Laboratory findings of LAD-I patients are excessive neutrophilic leukocytosis in the circulation and reduced expression or absence of CD18, CD11a and CD11b expression on

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leukocytes. Hematopoietic stem cell transplantation (HSCT) is the only curative treatment option for patients with LAD-I[7,9].

Here, we report clinical manifestations, immunological findings, medical approach, and outcome of 15 patients diagnosed with leukocyte adhesion deficiency type I at 3 different medical centers in Turkey. Moreover, we highlight the importance of molecular testing with a next-generation sequencing (NGS) targeted primary immunodeficiency panel and/or Sanger sequencing for establishing the genetic diagnosis, which is essential for family studies and genetic counseling.

Methods

Patients

Fifteen patients were diagnosed with LAD-I after referral to three Turkish clinical centers: Section of Pediatric Immunology, İhsan Doğramacı Children's Hospital, Hacettepe University, Ankara; Department of Pediatric Allergy and Immunology, Gaziantep University, Gaziantep; and Department of Pediatrics, Uludag University, Bursa. Demographical characteristics, clinical and laboratory findings of patients were recorded. Written informed consent was obtained from all participants.

Flow Cytometry Analysis of Peripheral Blood Samples

To measure the expression level of integrins on the leukocyte surface, flow cytometry analysis was performed with the following antibodies at optimal concentrations: anti-CD18-FITC and anti-CD11b-PC5. For the measurement of lymphocyte subgroups, peripheral blood mononuclear cells were stained with a mixture of the following antibodies at optimal concentrations: anti-CD3-FITC, anti-CD16-PE, anti-CD56-PE, anti-CD45-PerCP-Cy™5.5, anti-CD4-PE-Cy™7, anti-CD19-APC and anti-CD8-APC-Cy7. Finally, data were analyzed by FACS Canto II flow cytometer and FACS Diva software (BD Biosciences)

Genetic Analysis

Genetic analysis was performed at Hacettepe University and at Sanquin Research Institute in Amsterdam, Netherlands.

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Targeted next-generation sequencing was performed with either a primary immunodeficiency gene panel comprising 266 genes consisting of 5242 amplicons or a small LAD-panel consisting of five genes and 74 amplicons on the Ion Torrent PGM platform. Disease-related mutations were determined following the filtration steps (Combined Annotation Dependent Depletion (CADD), Genome Aggregation Database (gnomAD), Mutation Taster, etc.). Ion Reporter 5.16 Bioinformatics Software was used for characterization and filtering of variants. Variants with a coverage less than 5× for single nucleotide polymorphisms (SNPs) and 10× for small insertions and deletions (INDELs) were excluded. Common polymorphisms were discarded (Minor Allele Frequency, MAF>0.05) and exonic and nonsynonymous substitutions were selected. We also eliminated variants present in our in-house variant database (n = 600). Besides, we filtered rare variants (MAF<0.01) for presence in a comprehensive list of genes known to be involved in leukocyte adhesion deficiency pathogenesis (*ITGB2*, *FERMT3*, *SLC35C1*) or relevant pathways. Variants present in the 'Genome Aggregation Database Browser' (gnomAD) as homozygous state were ruled out. The remaining candidate variants were classified with three different variant function prediction tools (MutationTaster, PolyPhen2 and SIFT), following a manual checking step in IGV (Integrative Genomics Viewer). The alterations predicted as 'disease causing' and 'damaging' with all three prediction tools were selected. Among them, variants with a CADD score higher than 20 were chosen. Final candidates ensuring all the filtering criteria were defined as probable or definite disease-causing variant. All causative and candidate variants detected with primary immunodeficiency gene panel filtering steps were validated through Sanger sequencing.

Exons of the *ITGB2* gene were amplified under the following conditions: initial denaturation at 95 °C for 5 min, 35 cycles at 95 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, and final elongation step at 72 °C for 10 min. Obtained PCR products were purified with Exo-SAP (Thermo Scientific, USA). PCR products were sequenced with the appropriate primers and BigDye® Terminator v3.1 Cycle Sequencing Kits (Applied Biosystems). The products were loaded on Applied Biosystems 3130 Genetic Analyzer, and the results were analyzed with Sequencing Analysis v.5.2 software.

Statistical Analysis

Mean, median, minimum, and maximum values were calculated on IBM SPSS Statistics 23.

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Results

The study included a total of 15 patients (10 males (67%) and 5 females (33%)) from 15 unrelated families. Ten (67%) patients were Turkish and the other five were refugees of Syrian (n=4, 27%) and Iraqi (n=1, 7%) origin. Median age at onset of symptoms of the patients was 21 (6-180) days (mean 41 days). The median age of the patients at diagnosis was 3 (1-48) months (mean 10 months). Eleven (73%) patients had LAD-I diagnosis in their first 6 months. Fourteen (93%) patients had consanguineous parents. Demographic characteristics of patients with LAD-I are shown in **Table 1**.

Infections

Clinical findings observed in our patients in the follow-up are shown in Fig. 1. Delayed separation of the umbilical cord was present in 12 (80%) patients in our cohort. Eight (53%) patients presented with skin (n=4, 27%), soft tissue (n=3, 20%), perianal (n=2, 13.3%) or abdominal abscess (n=1, 7%). Viral infections such as EBV, CMV, Parainfluenza and/or Adenovirus were detected in 3 (20%) patients. Only 2 (13%) patients had signs of periodontal diseases such as gingivitis (n=1, 7%) and periodontitis (n=1, 7%). Diarrhea was present in 6 (40%) patients. Gram-positive bacteria (*Staphylococcus aureus, Staphylococcus epidermidis, Enterococcus faecium, Listeria monocytogenes*) and gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae*) were isolated from cultures. Fungal infections (*Aspergillus fumigatus, Candida albicans*) were detected in 20% (n=3) of the patients.

Complete Blood Count Analysis

Leukocytosis was observed in 14 (93%) patients at diagnosis, and leukocytosis with neutrophil predominance was observed in 11 (73%) patients. The median leukocyte and neutrophil counts of the patients were 44.7 (13.2-97.1)x10^3/μL (mean 45.2x10^3/μL) and 29.3 (5.4-82.1)x10^3/μL (mean 30.7 x10^3/μL), respectively. Detailed laboratory results of the patient without leukocytosis and three patients with leukocytosis but no neutrophil predominance were given in **Table 2**. Hemoglobin levels were lower than age-related reference values in 9 (60%) patients. Platelet counts of 10 (67%) patients were higher than reference values. C-reactive protein levels were higher than reference values in 12 (80%) patients.

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CD18 Expression Levels and Lymphocyte Subgroup Analysis

CD18 expression level was undetectable in 7 (47%) patients. It was ranging from 1% to 8% in 8 (53%) patients in our cohort. Cd11b expression was evaluated in 14 patients; it was undetectable in 5 patients (33%) and varying between 1-45% in 8 (53%) patients. However, one patient showed 99% CD11b expression on the cell surface, but CD18 expression of this patient was only 2%. Percentage of patients according to absolute numbers of lymphocyte subgroups compared to age-related reference values in patients are shown in **Fig. 2**.

Genetic Analysis

Genetic analysis of the *ITGB2* gene revealed 9 distinct mutations in 13 of the 15 patients with LAD-I (two patients had died before analysis). The same novel homozygous mutation, c.305_306delAA, was identified in all patients (n=4) of Syrian origin, from unrelated families. c.779_786dup is a novel homozygous mutation identified in a patient. Homozygous c.382G>A, c.382G>T, c.533C>T, c.562C>T, c.817G>A, c.1413_2080del and compound heterozygous c.533C>T and c.1777C>T mutations were identified in 8 patients. A homozygous c.533C>T mutation was identified in the patient showing 99% CD11b expression and 2% CD18 expression. All mutations identified in the patients are shown in **Table 3** and in **Fig. 3**, and the affected domains of CD18 are shown in **Fig. 4**.

Treatment and Outcome

Two (13%) of the patients died because of infections. Four (27%) patients underwent HSCT from a related donor. Two of these patients died because of HSCT complications, and the other two are alive and well. HLA typing has been done for nine patients. HSCT is planned for 3 (20%) patients who have matched related donors. Six (40%) of the patients have no matched related donor, and these patients are carefully being followed at our centers since their first admission.

Discussion

Leukocyte adhesion deficiency type I is a rare primary immunodeficiency that presents with typical clinical symptoms such as delayed umbilical cord separation and recurrent severe infections. The present report describes one of the largest comprehensive studies on clinical,

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immunological and molecular findings, and outcome of LAD-I patients from Turkey. Defects in CD18 expression in LAD-I lead to defective adhesion of leukocytes. Two forms of LAD-I have been described based on the level of CD18 expression on neutrophils: in severe form CD18 expression is <2% of normal, and in moderate form CD18 expression ranges from 2% to 30%[10]. Our patient cohort consisted of eleven patients with severe LAD-I and four patients with moderate LAD-I. Median age at the diagnosis of severe and moderate LAD-I cases were 3 (0.1-48) months and 5 (1-78) months, respectively, in a study from India[11]. The median age of our patients at diagnosis was 3 (1-48) months, and 73% (n=11) of patients had LAD-I diagnosis in their first 6 months. Parental consanguinity ratio was reported to be as frequent as 51% (in severe LAD-I) in studies from Israel[7] and India[11]. According to the Turkish Statistical Institute, ratio of consanguineous marriages in Turkey was 23% in 2012. In our cohort, the consanguinity ratio was 93%, indicating that autosomal recessive inheritance plays an important role, and consanguineous marriages contribute to occurrence of LAD-I.

In a study with 13 patients with LAD-I from Iran, all patients presented with leukocytosis and neutrophilia[12]. In our study leukocytosis was observed in 14 (93%) patients, and leukocytosis with neutrophil predominance was observed in 11 (73%) patients. In another study with 15 LAD-I patients from Iran, leukocyte counts showed persistent leukocytosis between 12.2 and 91x10^3/μL (median ~29.1 x10^3/μL), with neutrophil ratio 31–90%[13]. In our cohort, the median leukocyte and neutrophil counts of the patients were 44.7 (13.2-97.1)x10^3/μL (mean 45.2x10^3/μL) and 29.3 (5.4-82.1)x10^3/μL (mean 30.7x10^3/μL), respectively. These data indicate that persistent leukocytosis is a significant feature of LAD-I.

Delayed umbilical cord detachment was found in 8 (62%) out of 13 patients with LAD-I, and omphalitis was observed in 67% of the patients in a study from Israel[7]. Additionally, delayed separation of umbilical cord was observed in 5 (~63%) of 8 patients with LAD-I, and omphalitis was observed in only one patient in a study from Italy[14]. In the present study, signs of omphalitis were observed in approximately half of the patients, and delayed umbilical cord separation ratio was higher than in studies from Israel and Italy. This indicates that delayed umbilical cord detachment is one of the requisite components of LAD-I. De Rose et al. reported that patients with LAD-I present with numerous infections and autoimmune phenomena[14]. We did not observe autoimmune findings in our cohort except in a patient with alopecia areata. Periodontal manifestations such as oral ulcers, gingivitis and periodontitis have also been associated with

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LAD-I[2]. In the present study, periodontal destruction of the permanent dentition was observed in a 21-year-old male patient and gingivitis in a 4-year-old male patient. The reason for the low frequency of periodontal findings in our cohort might be the fact that most of the patients were in the infantile age group. In a study with 15 LAD-I patients from Iran, diarrhea was present in 5 patients, and hepatosplenomegaly was present in 4 patients[13]. Consistent with this study, diarrhea and hepatosplenomegaly were present in 6 and 3 patients, respectively, in our cohort.

In the study of De Rose et al., fungal infections were detected (*Aspergillus fumigatus* and *Candida parapsilosis*) in 2 out of 8 patients with LAD-I[14]. Besides, Wolach et al. reported that fungal infections of varying frequency and severity were recorded in about 3 (17%) out of 18 patients with LAD-I[7]. In our LAD-I cohort, 20% (n=3) of the patients had fungal infections. Interleukin-17 (IL-17)-mediated immunity has a significant role in protection from fungal infections, and IL-17 is abnormally upregulated in patients with LAD-I[15,16]. Severe periodontal disease and gingiva inflammation in the patients has been associated with elevated IL-17 levels as well[17]. IL-17 induction modulates protection against fungal infections via pro-inflammatory cytokines, secretion of antimicrobial proteins with candidacidal activities and neutrophil production and migration[15]. As β2 integrin–dependent neutrophil migration is impaired in patients with LAD-I, and antimicrobial proteins alone are not sufficient for fungal clearance, patients with LAD-I may present with fungal infections.

Wolach et al. reported that 11 out of 18 patients (61%) with LAD-I had chronic anemia[7]. In our cohort, 60% (n=9) of the patients had anemia, possibly related to chronic infections. Most of our patients (n=10, 67%) had thrombocytosis, which may have occurred secondary to an underlying inflammatory condition.

We have characterized 9 distinct variants in the *ITGB2* gene in 13 patients with LAD-I, of which 2 have not been reported previously. Interestingly, four unrelated patients from Syria had a novel c.305_306delAA mutation that might be a founder effect for LAD-I patients of Syrian origin. A homozygous novel c.779_786dup mutation was identified in one patient, and the CD18 expression in this patient was 1%. Homozygous c.382G>A, c.382G>T, c.533C>T, c.562C>T, c.817G>A and compound heterozygous c.1777C>T and c.533C>T mutations identified in 7 patients in our cohort were reported previously[18–23]. Large chromosomal deletions, including a whole *ITGB2* gene deletion and exonic deletions, were reported in several studies[3,23–26]. In our cohort, one patient harbored a large deletion (exon 12_14), which is probably the same as one

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reported previously[3]. The patient homozygous for the large exon 12_14 deletion had no detectable CD18 expression.

Cabanillas et al. reported a case of leukocyte adhesion deficiency type I in a 4-year-old girl with CD18 expression levels exceeding 30% but with a completely non-functional protein[27]. In our cohort, CD18 expression on cell surface of a patient homozygous for the novel c.305_306delAA variant was absent (clone: L130, BD). Interestingly, in repeated testing of the same patient, CD18 expression was found at higher expression levels (clone: 7E4, Beckman Coulter). This variability is related to characteristics of monoclonal antibody binding and incomplete protein expression. However, the clinical features and genetic findings of the disease were consistent with LAD-I. In any case, CD11a, CD11b and CD11c expression on the cell surface of the patient was 0%, 1% and 7%, respectively. On the other hand, in the other three homozygous patients with the novel c.305_306delAA variant, CD18 expression was only 1%. It must be considered that the results of flow cytometry analysis of CD18 expression in different laboratories may vary. CD18 expression level should be analyzed repeatedly by using different antibody clones in patients with clinical findings compatible with LAD-I and by considering the genetic cause and the truncated protein structure.

Gene therapy trials for patients with LAD-I are ongoing[28], but HSCT is the only curative treatment option of LAD-I currently available. HSCT success was 2/4 in our study. It is planned for 3 (20%) patients who have matched related donors. Six (40%) of the patients have no matched related donor, and these patients are carefully being followed at our centers since their first admission.

We must consider leukocyte and neutrophil counts of patients presenting with delayed detachment of the umbilical cord, omphalitis, and severe infections. Additionally, characterization of CD18 levels by flow cytometry analysis is essential for diagnosis, but molecular characterization of LAD-I is also important in terms of disease confirmation, therapeutic approach, prognosis, and follow-up of patients. Early diagnosis of the patients is critical in the management of the disease and genetic evaluation is essential for family studies and genetic counseling.

In conclusion, we report the clinical manifestations, immunological findings, treatment, and outcome of 15 patients diagnosed with LAD-I. Parental consanguinity was very high in the current study. Also, delayed umbilical cord detachment was observed more frequently than in

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previous reports. Additionally, two novel mutations were reported, extending the molecular spectrum of LAD-I.

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Authorship Contributions

All authors were involved in drafting the manuscript or revising it critically for intellectual content; all authors approved the final version to be published. IY performed experiments, collected the data, and wrote the paper. BO performed experiments, contributed to data collection. CT and SOH contributed to data or analysis tools. HNB, ESA and SE contributed to follow-up of the patients. SC, SSK and OK contributed to data collection and follow-up of the patients. KvL performed genetic analysis. DR supervised the study and corrected the manuscript. DC contributed to data collection and follow-up of the patients and supervised the study. IT conceived, designed, and supervised the study.

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Tables

Table 1. Demographic characteristics of patients with LAD-I

	Gender	Origin	Symptomatic Age (Days)	Age at Diagnosis (Months)	Parental Consanguinity
P1	M	Turkish	180	14	+
P2	M	Turkish	60	2	+
P3	F	Turkish	30	3	+
P4	F	Turkish	60	3	+
P5*	F	Turkish	12	3	+
P6*	M	Turkish	45	5	+
P7	M	Turkish	21	5	+
P8	F	Syrian	7	1	+
P9	M	Syrian	20	1	+
P10	M	Syrian	18	12	+
P11	M	Iraqi	30	4	+
P12	F	Syrian	105	48	+
P13	M	Turkish	10	48	-
P14*	M	Turkish	6	1	+
P15*	M	Turkish	16	2	+

***deceased**

Table 2. Laboratory results of the patient without leukocytosis and three patients with leukocytosis but no neutrophil predominance

	Neutrophil Count (x10^3/µL)	CD11b (%)	CD18 (%)	ITGB2 Gene Variant (cDNA)
P1*	5.4	7	8	c.533C>T and c.1777C>T
P3+	23.9	0	0	c.562C>T
P8+	23.2	3	1	c.305_306delAA

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P12+	19.6	1	0	c.305_306delAA

*the patient without leukocytosis.

+patients with leukocytosis but no neutrophil predominance.

Table 3. Mutations in *ITGB2* and CD18 expression in patients.

Patients	Zygosity	Exon	cDNA	Amino acid	Novelty	CD18 Expression
P1	Compound heterozygous	6 and 13	c.533C>T and c.1777C>T	Pro178Leu and Arg593Cys	Both reported	8%
P2	Homozygous	12_14	c.1413_2080del	-	Reported	0%
P3	Homozygous	6	c.562C>T	Arg188*	Reported	0%
P4	Homozygous	7	c.779_786dup	Thr263Cysfs*20	Novel	1%
P5	Homozygous	7	c.817G>A	Gly273Arg	Reported	0%
P6	Homozygous	6	c.533C>T	Pro178Leu	Reported	2%
P7	Homozygous	6	c.533C>T	Pro178Leu	Reported	0%
P8	Homozygous	4	c.305_306delAA	Lys102Serfs*39	Novel	1%
P9	Homozygous	4	c.305_306delAA	Lys102Serfs*39	Novel	1%
P10	Homozygous	4	c.305_306delAA	Lys102Serfs*39	Novel	1%
P11	Homozygous	4	c.382G>T	Asp128Tyr	Reported	3%
P12	Homozygous	4	c.305_306delAA	Lys102Serfs*39	Novel	0%
P13	Homozygous	4	c.382G>A	Asp128Asn	Reported	2%

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P14	n/a	n/a	n/a	n/a	n/a	0%
P15	n/a	n/a	n/a	n/a	n/a	0%

*n/a: not available.

Figure legends

Fig. 1. Clinical findings in patients with LAD-I. * Some of the patients have more than one clinical finding. The total number is not the number of patients.

Fig. 2. Percentage of patients according to absolute numbers of lymphocyte subgroups compared to age-related reference values.

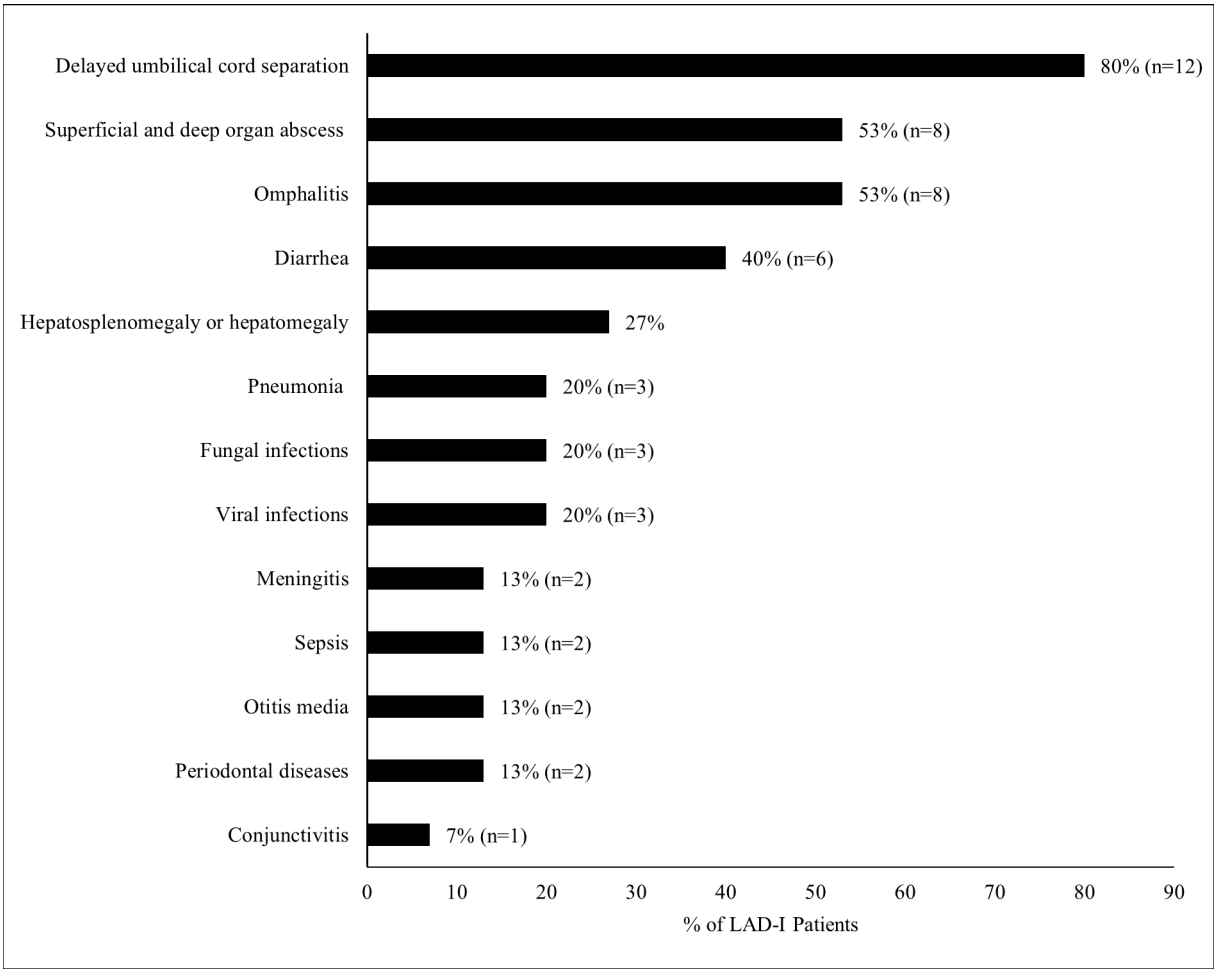
Fig. 3. cDNA of *ITGB2* gene and positions of mutations. *The figure was drawn according to NCBI Reference Sequence: NM_000211.5.

Fig. 4. Domain structure of the CD18 protein encoded by *ITGB2* and positions of mutations. VWFA, Von Willebrand factor type A domain; CYS, Cysteine-rich tandem repeat; TD, Transmembrane domain; CD, Cytoplasmic domain.

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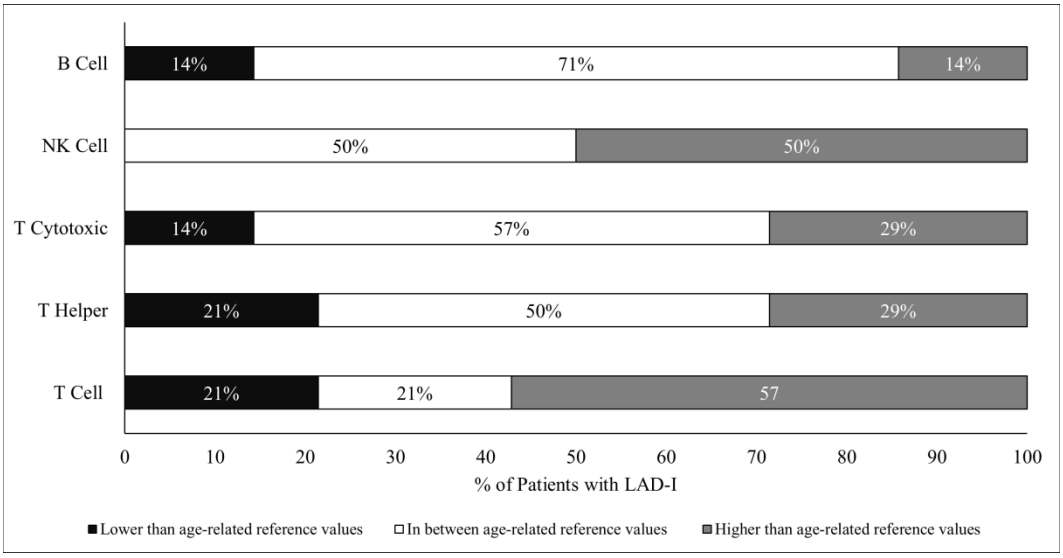
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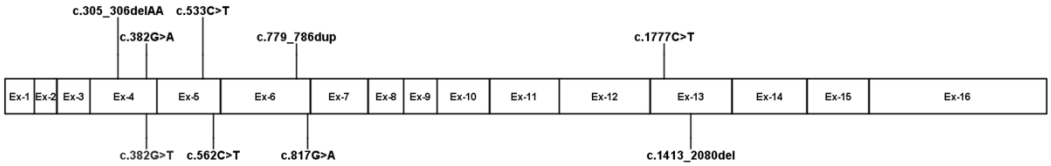
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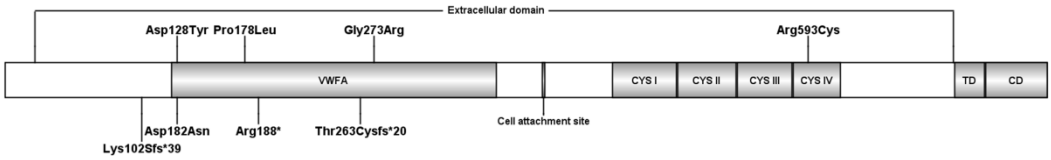
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