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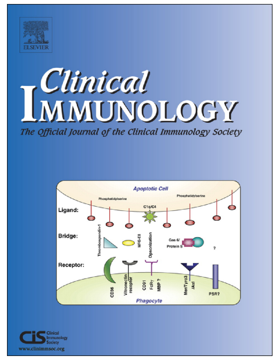

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Long term outcome of eight patients with type 1 Leukocyte Adhesion Deficiency (LAD-1): not only infections, but high risk of autoimmune complications.

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ABSTRACT

Leukocyte Adhesion Deficiency type 1 (LAD-1) is a rare primary immunodeficiency due to mutations in the gene encoding for the common β–chain of the β2 integrin family (CD18). Herein, we describe clinical manifestations and long-term complications of eight LAD-1 patients. Four LAD-1 patients were treated with hematopoietic stem cell transplantation (HSCT), while the remaining four, including two with moderate LAD-1 deficiency, received continuous antibiotic prophylaxis. Untreated patients presented numerous infections and autoimmune manifestations. In particular, two of them developed renal and intestinal autoimmune diseases, despite the expression of Beta-2 integrin was partially conserved. Other two LAD-1 patients developed type 1 diabetes and autoimmune cytopenia after HSCT, suggesting that HSCT is effective for preventing infections in LAD-1, but does not prevent the risk of the autoimmune complications.

Introduction

Leukocyte Adhesion Deficiency 1 (LAD-1, OMIM #116920) is a rare autosomal-recessive primary immunodeficiency, reported in fewer than 400 individuals, caused by a genetic defect in gene *ITGB2* (located at 21q22.3; OMIM *600065), encoding for the common β–chain of the β2 integrin family (CD18).[1] CD18 can form three heterodimers by interaction with the α subunits CD11a, CD11b (Mac-1), or CD11c; but the absence or abnormal synthesis of CD18 prevents leukocyte surface expression of CD11 subunits. Most of the *ITGB2* mutations lead to absent or decreased expression of CD18 on the cell surface as measured by flow cytometry, or more rarely, to nonfunctional expression of CD18 heterodimers, resulting in impaired leukocyte adhesion and migration[2].

LAD-1 patients display recurrent severe infections and impaired wound healing without pus formation. Hallmarks of the disease are moderate leukocytosis (in particular neutrophilia) in the absence of overt infection or leukocyte counts above 100,000/mL during acute infection and omphalitis, that is often associated with delayed separation of umbilical cord (usually after 3 weeks or later) [3]. Skin and soft tissue infections are also common. While patients with partial CD18 deficiency can develop in the second decade of life, gingivitis and periodontitis, sometimes associated with severe bone loss, and premature loss of primary and permanent teeth[4].

Two forms of LAD-1 have been described: the severe form, characterized by virtual absence of CD18 (CD18 expression below 2% expression of cells), and lethal outcome without hematopoietic stem cells transplantation (HSCT)[5], and the moderate form characterized by detectable CD18 expression at levels ranging from 2% up to 30% of cells, and longer survival without HCST [6]. Genetic analysis of LAD-1 patients revealed more than

86 mutations in *ITGB2* gene including missense, deletion, splice site, insertion and non-sense mutations [7].

Herein we report clinical manifestations, immune features and long-term outcome of LAD-1 patients depending on ITGB2 mutations, type of treatment that they received and the age of diagnosis.

Material and methods

Patients and study design

We describe eight patients (identified as P1, P2, P3, P4, P5, P6, P7, P8) with diagnosis of LAD-1 identified from 1993 to 2015, and followed at four Italian Clinical centers: Pediatrics Clinic, "ASST Spedali Civili" Hospital, University of Brescia; Department of Pediatrics, "Policlinico S. Orsola-Malpighi" Hospital, University of Bologna; Pediatric Hematology and Oncology Unit, "Policlinico Giovanni XXIII" Hospital, University of Bari "Aldo Moro", Bari; Department of Pediatrics, Institute for Maternal and Child Health "IRCSS Burlo Garofolo", Trieste. Medical history and clinical data were retrospectively obtained from medical records of the four clinical centers. On the basis of the age at which patients were identified, we analyzed long term outcome in those with diagnosis before three years of life (P1, P2, P3, P4, P5) or after three years of life (P6, P7, P8) (**Table 1**). All patients (or their caregivers in case of pediatric patients) signed an informed consent form according to the local ethical committee recommendations.

We obtained laboratory findings at diagnosis and at the last follow-up visit (see **Table 2** for leukocyte counts at the last follow-up visit in transplanted patients).

Molecular diagnosis

LAD-1 diagnosis was based on flow cytometry analysis of CD18 expression on neutrophils. Genetic analysis of ITGB2 was performed in patients with reduced CD18 expression. FITC-labeled or PE-labelled mAbs against CD18, CD11a, CD11b, CD11c were used in order to measure levels of β2 integrins expression on cell surface, as previously described[8]. For genetic analysis, DNA was extracted from peripheral blood leukocytes using standard techniques. Direct Sanger sequencing with primers spanning complete coding sequence and exon flanking regions was performed using the BigDye Terminator Kit (Applied Biosystems)and an ABI-Prism 310 sequencer (Applied Biosystems). Next, sequences were analyzed using the SeqScape software (Life Technologies). Mutations were designated as recommended by the Human Genome Variation Society (HGVS – http://www.hgvs.org) [9].

Statistical analysis

For statistical analyses, data are presented as numbers and percentages for categorical variables. Continuous variables are expressed as mean ± standard deviation (SD) if normally distributed (evaluated with D'Agostino-Pearson normality test) or, alternatively, as median ±interquartile range . Rank correlation test was used to analyze the correlation between leukocyte counts and the age at diagnosis. For all analyses a *p*-value <0.05 was considered statistically significant. Data were analyzed by MedCalc Software package for Windows, release 12.7 (MedCalc Software, Belgium).

Results

Patients

We describe eight patients with LAD-1 evaluated for11.19 ± 8.21 years . All the patients are alive, including four patients that have been treated with HSCT. Two patients are female, and six are male (see **Table 1**). Seven of the eight patients (87.5%: P2-P7) are Caucasian, one of African origin (P1). Median age at diagnosis was 0.856 years (IQ range 0.25 to 4.83). In five patients, diagnosis was established before three years of age (P1, P2, P3, P4, P5), while the remaining three patients were identified later in life (P6, P7, P8). Mean age at last follow-up visit was 13.86 ± 9.74 years. Leukocytosis was consistently observed in all subjects at diagnosis (41,168 ± 21,377 cells/mm^3 as average; ranging from 12,640 to 68,000 cells/mm^3). Analysis of leukocyte counts in LAD-1 patients showed higher leukocyte counts in patients with early-diagnosis as compared to those with delayed-diagnosis (respectively 55,006 ± 12,220 cells/mm^3 vs 10,107 ± 4,931 cells/mm^3; $p=0.036$). In addition, neutrophil counts were higher in patients with early diagnosis (38,406 ± 13,870 cells/mm^3 vs 13,460 vs 7,669 cells/mm^3, $p=0.036$) An inverse correlation was observed (r^2= 0.85; p=0.001) between leukocyte counts and age at diagnosis (**Figure 1**), suggesting that leukocyte counts can be moderately elevated in older children with LAD-1 deficiency.

CD18 expression and ITGB2 genetic analysis

Two out of eight patients showed partial expression of CD18 on cell surface (P5, P6) ranging from 5% to 60%; while CD18 was undetectable on leukocytes of the four patients (P1, P2, P3, P4, P8) or not evaluated in one subject (P7).

ITGB2 genetic analysis revealed eight distinct mutations (referral sequence ENST00000302347.9, see **Figure 2**). Mutations detected in patients P1, P3, P6 and P8 were novel, while the remaining four identified in patients P2, P4, P5, P7, P8 have been previously reported. In P1 and P3 we identified a deletion of 10 nucleotides (190-200del) resulting in frameshift (Gly40fsX49). Accordingly, CD18 expression by flow cytometry revealed no protein expression on cell surface. In P2, we detected an homozygous nonsense mutation in exon 3 (NM_000211.3: c.79A > T; NP_000202.2: p.Lys27X) that was previously reported by Fiorini M. et al., and that was associated with undetectable protein expression[10]. This mutation was associated with cytogenetic abnormalities of chromosome 21 (ring 21). In P4, we identified a homozygous mutation (c.[268delG];[268delG]) with undetectable CD18 expression [7]. In P5, we detected a homozygous missense mutation (c.1906T>C) that results in cysteine substitution with arginine (p.Cys612Arg)[11]. CD18 expression was reduced on cell surface, but still detectable (as low as 5% of cells). In P6 a new intronic homozygous mutation was found in the splicing site consensus sequence (IVS 15-2 A>G) leading to 5 amino-acid residues deletion (D750-K755del), and partial CD18 expression (60% of cells were CD18$^+$). In P7, we detected a non-sense homozygous mutation (A151T) with premature termination at codon L27[12]. In P8 we identified compound heterozygous mutations c.809C>T (p.A270V)/ c.819G>A (p.K294X), resulting in undetectable CD18 protein expression by flow cytometry.

Infections in LAD-1 patients

Five of the eight patients in our study presented delayed separation of umbilical cord (P1, P2, P4, P6, P7) during the perinatal period. Signs of omphalitis were observed only in P4, but not in the other patients.

Six of the eight patients (P1, P2, P5, P6, P7, P8) showed impaired wound healing and skin/soft tissue infections, presenting as perianal or pilonidal abscesses, fasciitis, pyodermitis, dactylitis. All patients had also other infectious manifestations such as upper respiratory tract infections (URTI), pneumonia, enteritis, urinary tract infections, otitis, osteomyelitis, that often required hospitalization for intravenous antibiotic therapy. Seven out of eight patients showed at least one episode of sepsis during their lifetime. Both Gram positive bacteria (*Streptococcus pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis, Clostridium difficile Enterococcus faecalis*) and Gram negative bacteria (*Pseudomonas aeruginosa, Escherichia coli, Klebsiella pneumonia, Proteus mirabilis, Enterobacter cloacae*) were isolated from cultures. Fungal infections were detected (*Aspergillus fumigatus* and *Candida parapsilosis*) in patients P7 and P2. Five of the eight patients (P1, P2, P5, P6, P7) had signs of periodontal diseases such as gingivitis and periodontitis, and complete tooth loss in one case (P7).

Despite the small sample size that limited statistical comparison among severe cases, we observed that patients that were not treated by Hematopoietic Stem Cell Transplantation (HSCT) because of the delayed diagnosis presented a higher number of infections as compared to patients who underwent HSCT, suggesting that HSCT constitutes the only cure for patients with LAD-1 [5]. As reported elsewhere[14], one of the patients (P6) was treated for one year with an ustekinumab, an antibody that target the p40 subunit that is shared by both interleukin-23 and interleukin-12 and inhibits interleukin-23-dependent production of interleukin-17.

Treatment and outcome

Treatment

Allogeneic HSCT was performed in four patients with early-diagnosis of LAD-1 and complete CD18 deficiency (P1, P2, P3, P4): P1 was transplanted with matched related sibling, P2 and P3 matched unrelated donors, or haploidentical donor (P4). Engraftment was successful in three patients (P1, P3, P4) while failed in patient P2, who was later transplanted in another European center. In particular, P1, P2, P3 were included in the multicenter report of HSCT outcome in LAD1 patients [5]. In these patients, cyclosporine A was used as immunosuppressive agent after transplant. Analysis of donors chimerism revealed that engraftment was full in patients P3 and P4, while was partial in P1 and P2[6]. In the other patients, no suitable donor has been found at the time of diagnosis or the family has refused this treatment. While in patient P8, HSCT has been proposed to the family and the search for a compatible donor is still ongoing. In these patients, several infections or other disease manifestations have been observed despite antibiotic prophylaxis (**Figure 3)**.

Auto[i]mmunity

All patients have been screened for autoantibodies, but they were detected in six out of the eight patients during their follow-up: they were detected in consecutive samples of three untransplanted patients (P5, P6, P7), but also in other three transplanted patients (P1, P2, P3). In P5, a patient with moderate LAD-1 not treated with HSCT, Crohn-like colitis and juvenile idiopathic arthritis were diagnosed at 12 of age, and successfully treated with the anti-TNF-α antibody infliximab[15]. At 16 years of age we observed the appearance of anti-nuclear antibodies (ANA), perinuclear anti-neutrophil cytoplasmic antibodies (pANCA), anti-beta 2 glycoprotein antibodies (B2GPI) and anti-cardiolipin antibodies. Elevated titers of anti-thyroglobulin antibodies (anti-TG) were identified in P6 (moderate type LAD-1 not transplanted patient) and P7 (severe type LAD-1 not transplanted patient), but anti-thyroid peroxidase (anti-TPO) antibodies were negative and thyroid hormones were normal. While low titers of anti-ANA (1:160 dilution) were detected in P3.

Autoimmune diseases were also observed in LAD-1 patients subjected to HSCT (P1, P2, P3), but these manifestations could constitute possible complications of the transplantation procedure. In particular, patient P1 developed type-1 diabetes mellitus (DM) 5 years after HSCT; onset of the disease was also associated with appearance of anti-islet cell antibodies (ICA), anti-insulin antibodies (IAA) and anti-glutamic acid decarboxylase (GAD-65) antibodies. While, patient P2 presented hemolytic anemia about 3 years after HSCT and thereafter autoimmune thrombocytopenia.

Renal involvement

Patient P5 presented proteinuria, suggestive of acute glomerulonephritis when she was 14 years old, but renal biopsy was refused . While P7 developed post-infectious glomerulonephritis with gross hematuria at the age of 14. At 30 years of age, an abdomen ultrasound performed despite normal serum creatinine, showed abnormal renal echotexture with loss of corticomedullary differentiation , as sign of early chronic kidney disease. An abdominal computed tomography (CT) scan was therefore performed, showing microcysts, but renal biopsy was not performed because patient refused further investigations.

Discussion

Although transplanted and untrasplanted patients cannot be compared in terms of autoimmune manifestations, an elevated risk of autoimmunity was observed in LAD-1 patients despite some of them were treated with HSCT [16]. Several other reports have shown that patients who received HSCT for hematological diseases can develop autoimmune diseases such as thyroiditis [17], type-1 DM [18, 19, 20], myasthenia gravis [21] and celiac disease [22].

How organ-specific autoimmune disease can develop in these patients is still unclear; it might be related to the conditioning regimen or, alternatively to impairment of the mechanisms controlling the tolerance. Interestingly, the intervals between transplant and onset of autoimmune diseases ranged from five months up to eight years, suggesting that risk of autoimmune complications can persist for many years in patients receiving HSCT. Patients with primary immunodeficiencies are often predisposed to autoimmunity because of impairment of mechanisms that regulate tolerance, such as defect of regulatory T cells [21,22,23,24]. In particular, we reported detection of autoantibodies in three out of four LAD-1 patients who underwent HSCT; which has been associated with autoimmune diseases in two of them. Indeed, in few cases, type-1 DM development has been observed in patients receiving HSCT[16, 18, 19, 20].

Interestingly, non-obese diabetic (NOD) mice that lack of integrin β2 (CD18, Itgb2) or integrin αL (CD11a, ItgaL) are protected against the risk to develop diabetes or insulitis because ItgaL null leukocytes cannot infiltrate the pancreatic islets of these mice[25]. This suggests that LAD-1 patients that are predisposed to DM can remain protected until CD18 deficiency is treated by HSCT. Once the expression of adhesion molecules is restored, donor's leukocytes could mediate the initiation and progression of pancreas-specific inflammation. This model could also apply to other autoimmune disorders, although different conditioning regimens could play an important role in the risk of developing autoimmunity after HSCT.

Despite there was previous evidence that LAD-1 patients are at risk of renal disease, several experimental models of renal injury suggest that many kidney disorders are related to dysregulation of leukocyte adhesion, and increased expression of beta2 integrins[26,27]. In particular, analysis of CD80 and CD18 expression by leukocytes derived from mice with nephrotic syndrome induced by doxorubicin showed an increase of CD18 expression in cytotoxic T lymphocytes, NK cells, and monocytes and higher CD80 expression in monocytes[26]. Moreover, kidney oxidative damage was positively correlated with CD80 expression in monocytes and with increase of creatinine serum levels, suggesting that drugs that interfere with integrin and costimulatory molecules might provide new therapeutic opportunities for treatment of renal injury. In another study by Tang et al., analysis of PMN-dependent proteinuria observed after intravenous injection of anti-glomerular basement membrane antibody in wild-type and Mac-1-deficient mice showed that nephritis was observed in wild-type animals but absent in Mac-1 mutant mice [27]. Therefore, LAD-1 patients with complete CD18 deficiency are probably protected against renal injury according to these models. However, two of our untransplanted patients (one of the early-diagnosed patients, P5, and one of the other group, P7) presented renal injury, suggesting that partial β2 integrins expression could lead to kidney damage in some patients.

In this study we show that early diagnosis of LAD-1 patients can result in better long term outcome in term of survival and prevention of infections for patients who are treated with HSCT. In contrast, severe or moderate LAD-1 patients who are not treated with HSCT experience higher numbers of infectious and autoimmune events in the course of their follow-up, suggesting that HSCT should always be proposed to patients with LAD-1 deficiency. Finally, an higher risk of autoimmune diseases was also observed in two patients receiving HSCT suggesting that careful long term monitoring of these patients is required even in HSCT-treated patients.

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Table 1. Clinical and genetic features of LAD-1 patients.

Patient (partial or severe deficiency)	Sex	Age at diagnosis (years)	Follow-up time (years)	Mutations in the CD18 alleles of patients	CD18 expression (%)	Clinical presentation and types of infections during follow-up	Identified bacteria and fungi	Treatment	Autoantibodies	
P1* (severe)	M	1.44	11.97	c.190-200del (GGCCCGGCTG); 190-200del (GGCCCGGCTG) p.G40fs*49; G40fs*49	<0.1%	Delayed separation of umbilical cord, Sepsis, Periodontal disease, URTI, Pharyngitis, Cold skin abscess	*Enterococcus faecalis, Escherichia coli, Pseudomonas aeruginosa, Streptococcus pneumoniae*	HSCT	Anti-ICA, GAD-65, IAA§	
P2* (severe)	M	0.27	4.21	c.[A79T];del p.L27*;?	<0.1%	Delayed separation of umbilical cord, Sepsis, Periodontal disease, URTI, Pneumonia, Cold skin abscess	*Enterobacter cloachae, Klebsiella pneumonia, Candida parapsilosis*	HSCT	Anti-Rh§	
P3* (severe)	M	0.23	20.50	c.190-200del (GGCCCGGCTG); 190-200del (GGCCCGGCTG) p.G40fs*49; G40fs*49	<0.1%	Sepsis, URTI, Pneumonia, Otitis media, Enteritis, UTI	*Enterobacter cloachae, Pseudomonas aeruginosa*	HSCT	ANA (1:160) §	
P4* (severe)	F	0.19	1.90	c.[268delG];[268delG] p.[D90fs*14];[D90fs*14]	<2%	Omphalitis, Sepsis, UTI	*Staphylococcus epidermidis, Escherichia coli*	HSCT	-§	
P5* (partial)	F	0.25	15.24	c.[T1834C];[T1834C] p.[C612R];[C612R]	5%	Sepsis, Periodontal disease, Mouth and tongue ulcers, URTI, Pneumonia, Enteritis, Perianal abscess and fistulas, Osteomyelitis	*Staphylococcus aureus, Proteus mirabilis*	Antibiotic prophylaxis	ANA, pANCA, B2GPI, anti-cardiolipin	
P6# (partial)	M	4.81	13.50	c.[2081-2A>G];[2081-2A>G] p.[D750-K755del];[D750-	60%	Delayed separation of umbilical cord, Sepsis,	*Enterococcus faecalis, Escherichia coli,*	Antibiotic prophylaxis	Anti-TG	

				K755del]		Aphthous stomatitis, Periodontal disease, Otitis media, Mastoiditis, Appendicitis Skin abscess, Perianal abscess, Pilonidal abscess	*Pseudomonas aeruginosa*			
P7[#] (severe)	M	4.01	21.76	c.[A79T];[A79T] p.[L27*];[L27*]	N/A	Delayed separation of umbilical cord, Sepsis, Aphthous stomatitis, Periodontal disease with severe bone loss, Otitis media, Enterocolitis, Appendicitis with peritonitis, Pilonidal abscess, Fasciitis, Pyodermitis, Dactylitis	*Clostridium difficile, Aspergillus fumigatus*	Antibiotic prophylaxis	Anti-TG	
P8[#] (severe)	M	4.90	0.44	c.[809C>T] ;[G819A] p.[A270V];[K294*]	<0.1%	Pneumonia, Perianal ulcer	Cultures not available	Antibiotic prophylaxis , waiting for HSCT	-	

* *Clinical diagnosis before 3 years of age;* [#] *Clinical diagnosis after 3 years of age;* [§] *Analysis of autoantibodies was performed after HSCT.*

Table 2. Leukocyte counts at the last follow-up visit in transplanted LAD-1 patients.

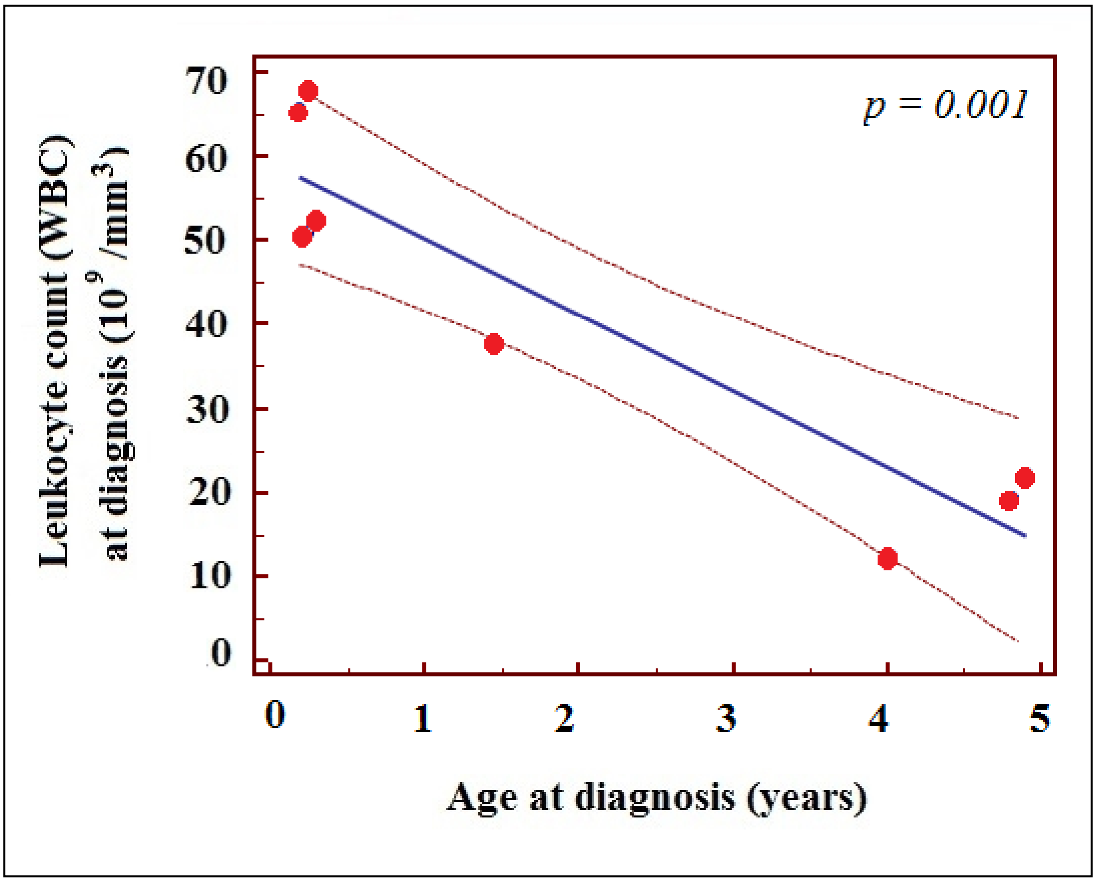
Patient	Age at HSCT (years)	WBC at last follow-up visit (cells/mm^3)	Neutrophils at last follow-up visit (cells/mm^3 and %)	Lymphocytes at last follow-up visit (cells/mm^3 and %)	Monocytes at last follow-up visit (cells/mm^3 and %)
P1	1.54	6,780	4,850 (71. 6 %)	1,350 (19.9 %)	325 (4.8 %)
P2	0.56	12,860	9,684 (75.3 %)	1,582 (12.3 %)	1505 (11.7 %)
P3	10.73	7,390	4,350 (58.9 %)	2,060 (27.8 %)	510 (6.9 %)
P4	0.25	17,260	5,178 (30 %)	10,700 (62 %)	690 (4 %)

Figure 1. An inverse correlation between leukocyte count and age at LAD-1 diagnosis. The graph reports leukocyte counts (cells/uL) on y axis of 8 LAD-1 patients at the age of diagnosis (x axis).

Figure 2. Domain structure of the β2 integrin (CD18) and location of ITGB2 mutations. Location of ITGB2 patients in 8 LAD-1 patients.

Figure 3. Clinical events in LAD-1 patients. The panels report the clinical events of 4 LAD-1 patients (P5, P6, P7, P8) that were not treated with HSCT.

Figure 1.



p = 0.001
Leukocyte count (WBC) at diagnosis (10⁹ /mm³)
70
60
50
40
30
20
10
0
0
1
2
3
4
5
Age at diagnosis (years)

Figure 2.

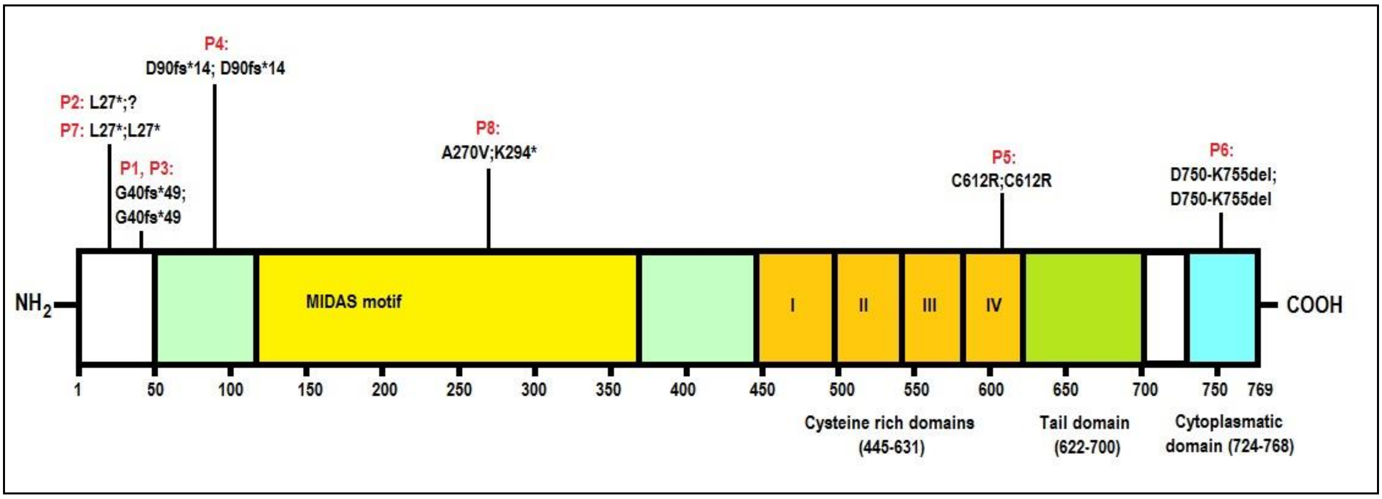
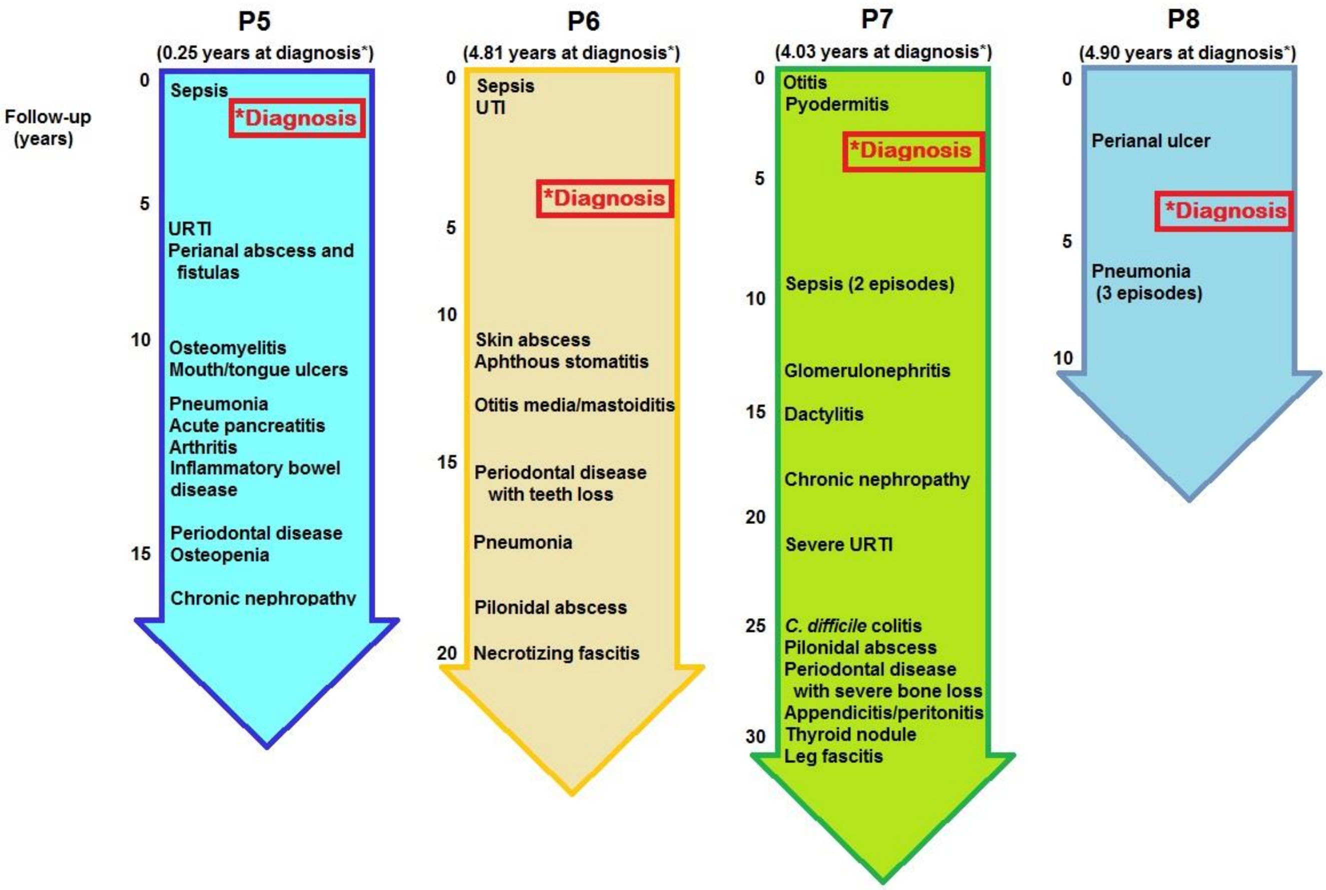

P4:
D90fs*14; D90fs*14
P2: L27*;?
P7: L27*;L27*
P1, P3:
G40fs*49;
G40fs*49
P8:
A270V;K294*
P5:
C612R;C612R
P6:
D750-K755del;
D750-K755del
NH2
MIDAS motif
I
II
III
IV
COOH
1 50 100 150 200 250 300 350 400 450 500 550 600 650 700 750 769
Cysteine rich domains
(445-631)
Tail domain
(622-700)
Cytoplasmatic
domain (724-768)

Figure 3


Follow-up (years)
P5
(0.25 years at diagnosis*)
0
Sepsis
*Diagnosis
5
URTI
Perianal abscess and fistulas
10
Osteomyelitis
Mouth/tongue ulcers
Pneumonia
Acute pancreatitis
Arthritis
Inflammatory bowel disease
Periodontal disease
15
Osteopenia
Chronic nephropathy
P6
(4.81 years at diagnosis*)
0
Sepsis
UTI
*Diagnosis
5
10
Skin abscess
Aphthous stomatitis
Otitis media/mastoiditis
15
Periodontal disease with teeth loss
Pneumonia
Pilonidal abscess
20
Necrotizing fascitis
P7
(4.03 years at diagnosis*)
0
Otitis
Pyodermitis
*Diagnosis
5
Sepsis (2 episodes)
10
Glomerulonephritis
15
Dactylitis
Chronic nephropathy
20
Severe URTI
25
C. difficile colitis
Pilonidal abscess
Periodontal disease with severe bone loss
Appendicitis/peritonitis
30
Thyroid nodule
Leg fascitis
P8
(4.90 years at diagnosis*)
0
Perianal ulcer
*Diagnosis
5
Pneumonia (3 episodes)
10