



# Transient neonatal diabetes mellitus and hypomethylation at additional imprinted loci: novel *ZFP57* mutation and review on the literature

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Received: 25 August 2018 / Accepted: 29 September 2018 / Published online: 13 October 2018  
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## Abstract

**Aim** 6q24-related transient neonatal diabetes mellitus (6q24-TNDM) is a rare imprinting disorder characterized by uncontrolled hyperglycemia during the first 6 months of life. The molecular etiology of 6q24-TNDM is attributable to overexpression of the paternally inherited *PLAGL1* and *HYMAI* genes located on the 6q24 locus. One of these major defects is maternal loss of methylation (LOM) at 6q24. In addition, approximately 50% of TNDM patients that present LOM at 6q24 can also display hypomethylation at additional imprinted loci (multilocus imprinting disturbances, MLID). Interestingly, the majority of these patients carry mutations in the *ZFP57* gene, a transcription factor required for the adequate maintenance of methylation during early embryonic development.

**Methods** Methylation analysis of 6q24 and additional imprinted loci was carried out by MS-MLPA in a Tunisian male patient with clinical diagnosis of TNMD. For the same patient, mutation analysis of the *ZFP57* gene was conducted by direct Sanger sequencing.

**Results** We report a novel nonsense mutation (c.373C>T; p.R125\*; ENST00000376883.1) at the *ZFP57* gene causing TNDM-MLID and describe detailed phenotype/epigenotype analysis of TNMD patients carrying *ZFP57* mutations.

**Conclusion** We provide additional support to the role of *ZFP57* as a genetic determinant cause of MLID in patients with TNMD.

**Keywords** Transient neonatal diabetes mellitus · Multilocus imprinting disturbances (MLID) · *ZFP57* gene · Tunisia

## Introduction

Neonatal diabetes mellitus can be subdivided into transient and permanent forms based on its clinical phenotype. It presents an incidence of 1 case in about 100,000 live births in European countries with the transient form accounting for about 50% (i.e., 1 in 200,000) [1]. Interestingly, different figures have been obtained in the Middle East with higher

prevalence of the permanent form [PMDM, 1:30,000 to 1:48,000 in Abou Dhabi and Anatolia (Turkey), respectively] compared with the transient form (TNDM, 1:350,000 in Abou Dhabi and 1:77,400 in Anatolia) [2, 3].

Transient neonatal diabetes mellitus (TNDM; OMIM#601410) is defined as a type of diabetes that appears during the first 6 months of life in a term infant and usually reverts before 5 months of age in most cases [4]. The cardinal presenting features of TNDM are hyperglycemia (which requires treatment with exogenous insulin), intrauterine growth retardation (IUGR), and variable degrees of dehydration [5].

TNDM is most frequently related to defects causing overexpression of the paternally expressed genes *PLAGL1* and *HYMAI*, located on human chromosome 6q24 (6q24-TNDM) [6, 7]. *PLAGL1* is a zinc finger DNA-binding protein with tumor suppressor activity and *HYMAI* is a non-coding RNA gene, whose function is currently unknown.

Managed by Massimo Porta.

**Electronic supplementary material** The online version of this article (<https://doi.org/10.1007/s00592-018-1239-3>) contains supplementary material, which is available to authorized users.

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Up-to-date, three major disease-causing mechanisms have been described for this disorder: (1) paternal uniparental disomy of chromosome 6 [UPD(6)pat] (40%); (2) paternal duplications of 6q24 (32%); and (3) maternal loss of methylation (LOM) at the *PLAGL1* differentially methylated region (*PLAGL1*:alt-TSS-DMR) (28%) [8]. In addition, patients with LOM at *PLAGL1*:alt-TSS-DMR may also present methylation defects at additional imprinted loci (multilocus imprinting disturbances, MLID); mostly affecting *GRB10*:alt-TSS-DMR (7p12.1), *PEG3*:TSS-DMR (19q13.4), and *PPP1R13L* (19q13.32), while *MEST*:alt-TSS-DMR (7q32.2), *KCNQ1OT1*:TSS-DMR (11p15.5), *GNAS-ASI*:TSS-DMR (20q13.3), *NAP1L5*:TSS-DMR (4q22.1), *SNU13*:alt-TSS-DMR (22q13.2), *WRB*:alt-TSS-DMR(21q22.2) and *PPIEL*:Ex1-DMR (1p35.3) are hypomethylated in some patients [9–12]. In a proportion of these MLID patients, recessive mutations in the *ZFP57* gene have been identified. The *ZFP57* gene, located at 6p22.3, is a transcription factor involved in the correct maintenance of methylation of imprinted loci [13, 14].

In this study, we present the case of a TNMD patient with 6q24 LOM, MLID and a novel nonsense mutation in the *ZFP57* gene. We also review data from the literature and discuss the clinical picture and molecular basis of our patient, highlighting the role of *ZFP57* as an important genetic determinant for the diagnosis of TNMD-MLID.

## Methods

### Case report

The patient was the second child of a young consanguineous couple. The mother had a first pregnancy, poorly followed, giving birth to an anencephalic premature baby who died 24 h later. The patient was born by caesarean section at 37 weeks of gestation due to acute foetal distress after an uneventful pregnancy. He was small for gestational age, weighting 2380 g (between 3rd and 10th percentile), with a length of 47 cm (25th percentile) and a head circumference of 34 cm (50–75 percentile). He was left in maternity near his mother. His blood glucose level (BGL) was checked using Bedside reagent test-strip glucose. BGL was high (5 g/l) after 1 h of life, confirmed by laboratory analysis (27.8 mM of glucose). The neonate was then admitted in neonatology department. On examination, there were no particular physical findings, except a mild dysmorphism consisting in macroglossia, large protruding tongue, and nasal bridge. Unilateral cryptorchidism was also noticed. Thyroid function testing, performed because of the protruding tongue, showed elevated TSH level on the 5th day of life (9 mUI/l) alongside normal T4 levels (14 pmol/l). Thyroid function test showed normal values

of TSH (3.4 mUI/l) 15 days later. The blood gasometry, blood cell count, and C reactive protein levels were normal on admission, 24 h and 48 h later, which ruled out a possible maternofetal infection. The transfontanelar sonography and abdominal ultrasound test did not reveal any abnormalities. The echocardiography and karyotype analysis were normal. Islet cell antibodies were negative. Upon hospitalisation, adequate glycemic control was achieved using continuous intravenous infusion of standard insulin with an initial rate 0.06 U/kg/day via central venous catheter. Insulin doses were increased up to 0.1 U/kg/day. Blood ketones were negative at hyperglycemic periods with no episodes of acidosis. A progressive normalisation of glycemia was observed. Insulin need declined gradually and normoglycemia was obtained with insulin withdrawal at 2 months of age, while he was still hospitalised. Within the first 3 months, BGL values ranged between 0.8 and 1.5 g/l; then, at the fourth month, they stabilised and never exceeded 1 g/l. The patient is currently 4 years of age; his growth and physical development are normal.

### Genetic analyses

Genomic DNA was extracted from the peripheral blood of the patient and his parents using the FlexiGene DNA Kit (Qiagen, Hilden, Germany) after the informed written consent was signed. This study has been approved by the national Ethics Committee at Farhat HACHED University Hospital (Tunisia).

### Methylation analysis

Methylation analysis was used to detect the methylation status at 6q24 using the SALSA MS-MLPA<sup>®</sup> ME032 kit (MRC-Holland, Amsterdam, The Netherlands) according to the manufacturer's instructions. Polymorphic microsatellite analysis including *D6S286*, *D6S262*, *D6S472*, *D6S976*, *D6S314*, *D6S311*, *D6S308*, and *D6S305* (<http://genome.ucsc.edu/>) was performed by fluorescent PCR.

Methylation analysis of additional DMRs was also performed using SALSAMS-MLPA<sup>®</sup> ME030, ME031, and ME028 kits (MRC-Holland, Amsterdam, The Netherlands). The studied loci included the 11p15.5 region, the *GNAS* locus (20q13.3), as well as the promoter regions associated with *SNRPN* (15q11.2). The products were analyzed with the ABI PRISM 3500 DNA Analyzer (Applied Biosystem). MS-MLPA results were then confirmed by methylation-sensitive single-nucleotide primer extension (MS-SNuPE) as previously reported [15].

## ZFP57 gene sequencing

*ZFP57* gene was analyzed by direct sequencing of exons 1–6 and flanking exon/intron boundaries in the patient and his parents (Transcript: ENST00000376883.1). The primers and conditions are available on request.

For phenotype–genotype correlations, the effect of previously described non-synonymous variants was calculated using in-silico softwares such as PolyPhen-2 and Mutation Taster.

## Results

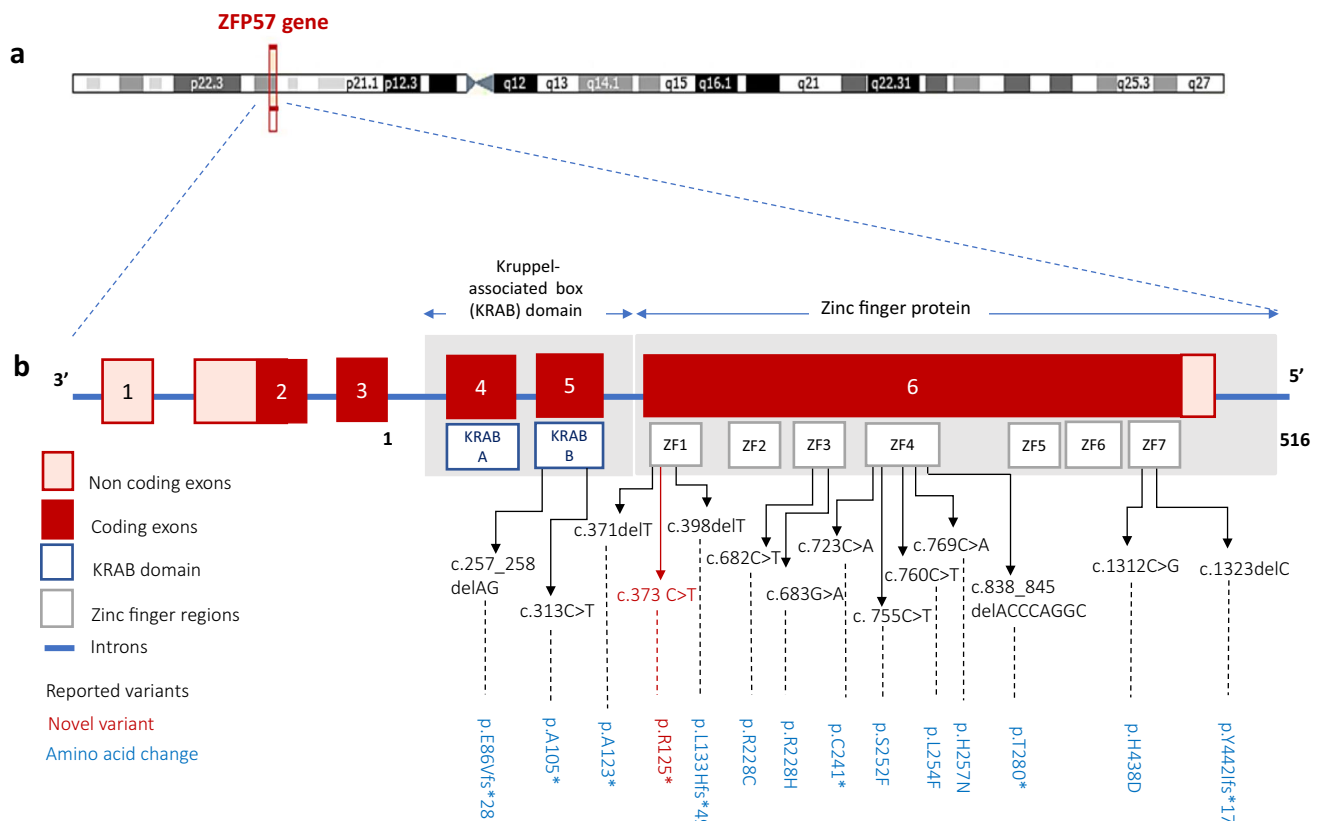
### Methylation analysis

Initial methylation testing of *PLAGL1*:alt-TSS-DMR(6q24) using MS-MLPA revealed a complete loss of maternal methylation. Microsatellite analysis of the patient and his parents around 6q24 region showed biparental inheritance and confirms the diagnosis of TNDM due to 6q24 hypomethylation (supplementary Table 1). To extend these findings, DNA methylation was then analyzed at other imprinted loci across the genome. In addition to

the *PLAGL1*:alt-TSS-DMRLOM, the patient had MLID: he presented total LOM at *GRB10*:alt-TSS-DMR and partial LOM at *MEST*:alt-TSS-DMR, *GNAS-XL*:Ex1-DMR, and *GNAS A/B*:TSS-DMR. No aberrant methylation was found at the remaining studied DMRs. Results were confirmed by the MS-SNuPE assay.

### ZFP57 analysis

Sanger sequencing of *ZFP57* showed a novel homozygous coding variant c.373C>T in exon 6 which generated a premature stop codon (p.Arg125\*). The same mutation was found in heterozygosity in both parents. Structure and domains of *ZFP57* protein are illustrated in Fig. 1. Up-to-date, 14 *ZFP57* gene mutations (six missense, five frameshift, and three nonsense mutations) have been reported. Table 1 summarises previously reported mutations and their expected functional effects. In-silico predictions of missense mutations (p.H438D, p.R228H, p.H257N, p.R228C, p.L254F, and p.S252F) revealed them to be probably damaging on PolyPhen-2v.2.2.2. Online tool Mutation Taster also revealed these mutations to be ‘disease causing’.



**Fig. 1** **a** Diagrammatic representation of cytogenetic location of *ZFP57* on chromosome 6. **b** Landscape of *ZFP57* mutation spectrum—all reported variants up-to-date (in blue) and the novel variant found in our patient (in red). (Color figure online)

**Table 1** *ZFP57* mutations identified to date in TNDM-MLID patient and their respective in-silico prediction scores

References	Mutation (Type, Zygosity)	Genotype		In-silico prediction score (reported/current) <sup>a</sup>	
		Variant	Amino acid change	PolyPhen2	Mutation Taster
Mackay et al. [9]	Nonsense, homozygous	c.723C>A	p.C241*	–	Disease causing
	Frameshift, homozygous	c.257_258delAG	p.E86Vfs*28	–	Disease causing
	Frameshift, homozygous	c.1323delC	p.Y442Ifs*17 (previously named p.G441Gfs*17)	–	Disease causing
	Missense, homozygous	c.1312C>G	p.H438D	0.991	Disease causing
				Probably damaging	
	Missense, homozygous	c.683G>A	p.R228H	0.982	Disease causing
				Probably damaging	
Boonen et al. [13]	Missense, homozygous	c.769C>A	p.H257N	1.00	Disease causing
				Probably damaging	
	Frameshift, compound heterozygous	c.838_845 delACCCAGGC	p.T280* (previously named p.279fs*1)	–	Disease causing
Court et al. [19]	Frameshift, compound heterozygous	c.398delT	p.L133Hfs*49	–	Disease causing
	Missense, homozygous	c.760C>T	p.L254F	1.00	Not damaging
				Probably damaging	
Boyraz et al. [20]	Missense, homozygous	c.682C>T	p.R228C	1.00	Not damaging
				Probably damaging	
Iyigun et al. [21]	Frameshift, homozygous	c.371delT	p.A123*	–	Disease causing
Current case	Missense, homozygous	c.755C>T	p.S252F	1.00	Disease causing
				Probably damaging	
	Nonsense, homozygous	c.313C>T	p.A105*	–	Disease causing
	Nonsense, homozygous	c.373C>T	p.R125*	–	Disease causing

<sup>a</sup>Missense variants effect was calculated using in-silico prediction softwares as PolyPhen-2 and Mutation Taster

## Epigenotype–phenotype correlations

Comparison of the clinical and (epi)genetic features of our patient and all reported individuals with *ZFP57*-related TNDM-MLID are summarised in Table 2. All affected individuals were ascertained through presentation of IUGR 28/29 (96%), hyperglycemia 28/30 (93%), and macroglossia 17/29 (63%) as key clinical features. The high rate of IUGR seems to be related to the crucial role of insulin in foetal growth. Various combinations of congenital anomalies were observed in *ZFP57*-related TNDM-MLID patients, including facial dysmorphism in 9/30 (30%), cardiac anomalies in 7/29 (24%), umbilical hernia in 7/30 (23%), and developmental delay in 5/29 (17%). Epilepsy and structural brain abnormalities occurred occasionally in 2/28 (7%) and 2/30 (6%) of patients. Furthermore, patients with TNDM-MLID are less likely to develop ketoacidosis, reported in only one newborn.

Genetically, notable similar methylation anomalies were observed in the affected individuals with *ZFP57* gene mutations. The epigenotype always included loss of methylation at the TNDM locus and variable combinations of

hypomethylation at other maternally imprinted loci: the most affected DMRs are *GRB10*:alt-TSS-DMR (18/30; 60%) and *PEG3*:TSS-DMR (17/30; 57%). LOM at *MEST*:alt-TSS-DMR, *KCNQ1OT1*:TSS-DMR, and *GNAS-AS1*:TSS-DMR is less common.

So far, there have been 14 mutations described at *ZFP57*, including both missense and truncating variants. Most of them are located at the third and fourth zinc finger domains.

There seems to be no correlation between the mutation type or its location and the methylation profile (including affected DMRs and the level of the methylation defect). Regarding the phenotype, it is not related to the mutation, neither the number of affected DMRs nor the methylation levels.

## Discussion

TNDM, as uncontrolled hyperglycemia during the first 6 months of life, is an extremely rare presentation of diabetes [1–3]. Even some genetic defects have been associated with the disease, it is mainly caused by epigenetic defects at

**Table 2** Review of clinical and epigenetic features of individual with TNDM-MLID with *ZFP57* mutations

N	Current case	Boonen et al. [13] <sup>b</sup>	Court et al. [19]	Docherty et al. [4]	Boyras et al. [20]	Iyigun et al. [21]	Summary	
	1	14	1	12	1	1	30	%
<b>Clinical features<sup>a</sup></b>								
Hyperglycaemia	1/1	12/14	1/1	12/12	1/1	1/1	28/30	93%
IUGR	1/1	12/13	1/1	12/12	1/1	1/1	28/29	96%
Macroglossia	1/1	8/11	1/1	7/12	0/1	0/1	17/27	63%
Facial dysmorphism	0/1	4/13	1/1	4/12	0/1	0/1	9/30	30%
Umbilical hernia	0/1	3/13	1/1	3/12	0/1	0/1	7/30	23%
Development delay	0/1	5/13	0/1	0/12	0/1	0/1	5/29	17%
Congenital heart disease	0/1	4/13	0/1	3/12	0/1	0/1	7/29	24%
Epilepsy	0/1	2/12 <sup>c</sup>	0/1	0/12	0/1	0/1	2/28	7%
Structural brain abnormalities	0/1	2/14	0/1	0/12	0/1	0/1	2/30	6%
Genital abnormalities	1/1	0/14	1/1	0/12	0/1	0/1	2/30	6%
Ketoacidosis	0/1	0/14	0/1	0/12	0/1	1/1	1/30	3%
Hypothyroidism	1/1	0/14	0/1	0/12	0/1	0/1	1/30	3%
<b>Epigenetic features</b>								
<i>PLAGL1:alt-TSS-DMR</i>	1	14	1	12	1	1	30/30	100%
<i>GRB10:alt-TSS-DMR</i>	1	14	1	NA	1	1	18/30	60%
<i>PEG3:TSS-DMR</i>	0	14	1	NA	1	1	17/30	57%
<i>MEST:alt-TSS-DMR</i>	1	3	1	NA	0	0	5/30	17%
<i>KCNQ1OT1:TSS-DMR</i>	0	3	0	NA	0	0	3/30	10%
<i>GNAS-AS1:TSS-DMR</i>	1	5	1	NA	1	0	8/30	27%

N number of patients, IUGR intrauterine growth retardation, DMR differentially methylated region, NA non-available

<sup>a</sup>In the “Clinical features” section, figures represent number of individuals with feature present/number of individuals with clinical information available for that feature

<sup>b</sup>9 and 12 affected individuals were reported by Mackay DJG et al., 2008

<sup>c</sup>Two affected individuals had a single seizure neonatally or in childhood

6q24 [7]. Despite their common epimutation, 6q24 LOM, 50% of TNDM patients have MLID and a portion of them are associated with mutations of the *ZFP57* gene [9].

*ZFP57* gene is localized on chromosome 6p22.1. It is comprised of 6 exons and encodes a protein of 516 amino acids (ENST00000376883.1). The *ZFP57* protein contains a Kruppel associated box (KRAB) domain encoded by exons 4 and 5 and seven zinc fingers (ZFs) domains encoded by exon 6 (Fig. 1) [9, 13]. The previous studies have demonstrated the critical role of *ZFP57* in the maintenance of DNA methylation at multiple germline DMRs [13, 14]. Mouse and human *ZFP57* proteins bind the methylated hexanucleotide TGC<sup>met</sup>CGC [16]. It has been suggested that, in mouse, the protein recognises the methylated TGC<sup>met</sup>CGC target sequence using the first and the second ZFs, while specific recognition of the same methylated sequence in human is made through the third and the fourth ZFs [17, 18].

To date, 14 *ZFP57* variants have been identified in relation to TNDM-MLID, including the novel mutation described here (Table 2) [4, 9, 13, 19–22]. Eight of these *ZFP57* mutations [p.E86Vfs\*28, p.A105\*, p.A123\*,

p.R125\*, p.L133Hfs\*49, p.C241\*, p.T280\* (previously named p.279fs\*1), and p.Y442Ifs\*16 [previously named p.G441Gfs\*17)] generate truncated forms of the protein unable to bind the methylated hexanucleotide TGC<sup>met</sup>CGC, while six are missense mutations (p.R228C, p.R228H, p.S252F, p.L254F, p.H257N, and p.H438D). Functional studies conducted by Baglivo et al. described the effect of some of the reported *ZFP57* mutations on protein structure [18]. In the majority of the cases, truncated and missense mutations affect ZF3 and ZF4 regions, though genetic variants have been also detected at the different domains in patients with 6q24-TNDM. Sequence analysis of hypomethylated regions in *ZFP57*mut/mut individuals revealed enrichment in TGC<sup>met</sup>CGC motif of five maternally imprinted regions: *PLAGL1:alt-TSS-DMR*, *GRB10:alt-TSS-DMR*, *PEG3:TSS-DMR*, *GNAS-AS1:TSS-DMR*, and *NAP1L5:TSS-DMR* [8], the most affected DMRs in TNDM-MLID. At a phenotypic level, TNDM-MLID individuals carrying *ZFP57* mutations show no clear genotype–phenotype correlation, which explains the variable clinical presentation of this disease [13]. It is, therefore, difficult to predict the

phenotype in individuals with MLID, even if compared with patients with hypomethylation confined to 6q24 they present a higher frequency of congenital abnormalities [17]. Table 1 summarises the percentages of occurrence of clinical and (epi)genetic features of the reported TNDM-MLID patients with *ZFP57* mutations. Most patients are diagnosed with hyperglycemia (and associated IUGR); commonly reported congenital abnormalities are macroglossia, facial dysmorphism, cardiac anomalies and umbilical hernia [4, 9, 13, 19–22].

Epigenotype–phenotype correlation, that may explain the clinical variability observed in some TNDM-MLID, was discussed by Boonen et al. [13]. Two possible mechanisms were proposed: the affected loci and the divergent levels of hypomethylation at each of them. However, no obvious relationship between both mechanisms and the phenotype was found in their studies [10]. Our data were broadly in line with these results: genetically, notably similar methylation anomalies were observed in our TNDM-MLID patient with total LOM at *PLAGL1*:alt-TSS-DMR and *GRB10*:alt-TSS-DMR and partial LOM at *MEST*:alt-TSS-DMR, and *GNAS-AS1*:TSS-DMR. However, apart from TNDM-DMR LOM associated with hyperglycemia and IUGR, no consistent abnormalities attributed to changes at *GRB10*:alt-TSS-DMR, *MEST*:alt-TSS-DMR and *GNAS-AS1*:TSS-DMR were found. Regarding the divergent levels of hypomethylation, we found no obvious correlation between methylation levels of affected loci and neither the phenotype. Extending the hypothesised mechanisms, we did not find correlation between the phenotype and the mutation type and its location (affected zinc finger regions).

Besides the implication of epigenetic defects at 6q24 as major finding, TNDM can be associated with some additional genetic defects [7]. Activating heterozygous mutations in the genes encoding either of the subunits of the ATP-sensitive  $K$  channel ( $K_{ATP}$  channel; *KCNJ11* or *ABCC8*) of the pancreatic beta-cell are the second most common cause [22], followed by recessive activating mutations at *INS* [23] and some very rare cases with genetic alterations at *SLC2A2* [24] or *HNF1B* [25].

The identification of the molecular underlying mechanism is very important as, for example, even 6q24-TNDM is a disorder diagnosed in infancy, a long-term follow-up is important during adolescence and early adulthood as about 50% of patient with 6q24-related TNDM may have a relapse to a permanent form of diabetes mellitus during these periods due to increased insulin demand [4]; the genetic counseling varies if they present only epigenetic changes (with no recurrence) or alterations at *ZFP57* (with a recurrent risk on 25% if both parents are carriers); those patients with alterations at  $K_{ATP}$  channel are susceptible of treatment with sulfonylureas [26], patients carrying alterations at *HNF1B* are susceptible

to present renal cysts among other disorders [27] or Fanconi–Bickel Syndrome if associated with *SLC2A2* [28].

To conclude, *ZFP57* is an important determinant for the diagnosis of TNDM as well as a crucial factor for understanding the genetic drivers of MLID. Therefore, *ZFP57* analysis is advised for accurate genetic counseling. Gathering clinical and (epi)genetic data on TNDM-MLID patients carrying *ZFP57* mutations, *ZFP57* sequencing should be considered in any TNDM case when at least three DMRs are affected, mainly *PLAGL1*:alt-TSS-DMR, *GRB10*:alt-TSS-DMR, and *PEG3*:TSS-DMR (19q13.4).

## Compliance with ethical standards

**Conflict of interest** The authors declare that they have no competing interest.

**Ethical approval** Ethical procedures followed were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national, local ethics committee: Comitato Etico Centrale IRCCS Lazio, Sezione IFO/Fondazione Bietti, Rome, Italy) and with the Helsinki Declaration of 1975, as revised in 2008.

**Informed consent** Informed consent was obtained from the patient included in the study.

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