CASE SERIES

Exploring the severity and early onset of familial type 1 diabetes in Romania: genetic and microbiota insights

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ABSTRACT

Type 1 diabetes mellitus (T1DM) is a chronic condition characterized by pancreatic autoimmunity and destruction of the insulin producing beta-cells. The risk of familial type 1 diabetes (FT1DM) is greater in families with paternal T1DM. The children with paternal FT1DM have a more severe form of the disease with diabetic ketoacidosis. Three families with FT1DM, out of which two with paternal diabetes and daughters diagnosed with this disease, and one family with sibling FT1DM were evaluated between 2019-2021 in the Pediatric Diabetes and the Diabetes, Nutrition and Metabolic Departments of a tertiary hospital. Clinical, biological, and genetic evaluations were performed, together with an assessment of the gastrointestinal microbiota. The Romanian children with FT1DM had a more severe onset, a median of age at onset of 9 years old and a genetic predisposition with positive HLA DR3/R4, DQB1*02:01. The protecting allele, DPB1*04:01, was found only in the siblings with FT1DM. A gastrointestinal dysbiosis, characterized by pro-inflammatory bacteria, with high levels of *Enterobacteriaceae* and *Candida*, was observed in the gut microbiota. This is the first case series of FT1DM in Romanian patients that shows the presence of genetic determinants but also a pathological microbiota which may determine a more severe and an early-age onset of disease.

KEYWORDS: familial type 1 diabetes; pediatric diabetes; case series; microbiota

INTRODUCTION

Type one diabetes mellitus (T1DM) is a chronic autoimmune disease characterized by pancreatic autoimmunity which targets the insulin producing beta-cells. There is an important genetic influence involved both in the etiology of the disease, and in the clinical manifestations. Thus, children with an affected family member have a 5% risk of developing this disease by the age of 20. The risk of developing familial type 1 diabetes mellitus (FT1DM) is reported to be double to triple in families with a T1DM positive diagnosis in the father, rather than in the mother [1-5].

A more severe metabolic onset characterizes the patients with paternal FT1DM, the offspring have more severe clinical manifestations, as diabetic ketoacidosis, and a younger disease onset, versus the maternal cases. Studies also show that these patients have a greater weight loss and a longer duration of symptoms prior to diagnosis [3,6,7].

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In Caucasian populations, two HLA haplotypes are known to have strongest genetic risk factors, respectively DRB1*03:01-DQA1*05:01-DQB1*02:01 (known as DR3-DQ2) and DRB1*04-DQA1*03-DQB1*03:02 (known as DR4-DQ8). The allele associated with the highest risk is DRB1*04:05, followed by DRB1*04:01, DRB1*04:02 and DRB1*04:04, while DRB1*04:03 shows protective effects [8].

Besides the genetic transmission, the involvement of gastrointestinal microbiota is hypothesized to be the cause of T1DM, with a reduction in microbiota richness, an increase of *Bacteroides* and a low abundance of butyrate-producing species. A negative correlation was observed among butyrate-producers, intestinal permeability, and the risk of T1DM development. Even during the late phase of prediabetes, a reduced number of butyrate producers were identified, indicating the microbiota's influence on beta-cell autoimmunity and disease progression [9-11].

A study which combined metagenomics and metabolomics approaches examined T1DM patients at onset, their siblings, and healthy individuals. This investigation revealed an increase in *Clostridiales* and *Dorea*, along with a decrease in

Dialister and *Akkermansia* in patients with this disease but also in their siblings. Elevated levels of certain metabolites and microbial taxa such as isobutyrate, malonate, *Clostridium, Enterobacteriaceae, Clostridiales*, and *Bacteroidales* were observed. These findings provide insights into distinct gut microbial and metabolic signatures associated with the progression and severity of T1DM [9,12].

Furthermore, alterations in the gut microbiota composition of T1DM patients have been linked to glycemic control and diabetes-related complications, suggesting a potential role of the gut microbiota in the development of diabetic complications [13-15].

The purpose of this study is to emphasize the importance of paternal genetic transmission of T1DM in the families of patients with this disease, but also to stress the importance of environmental factors on the genetic determinants that might cause the clinical disease onset.

CASE SERIES REPORT

Three recently diagnosed T1DM patients (within 6 months after diagnosis) and their 1st degree T1DM family member, were evaluated in the Pediatric Diabetes and the Diabetes, Nutrition and Metabolic Departments between 2019-2021, for the initiation of a state reimbursed continuous glucose monitoring system. All three were girls, with Romanian Ethnicity and from an urban environment. Two of them, patient 1 (9 years old) and patient 2 (8 years old), have a father with diabetes, whilst patient 3 (10 years old) has a male sibling with T1DM. The genetic penetrance of FT1DM in the 3 recent cases are showed in Figure 1.

From their medical history all girls had cesarean birth, and the fathers of the children and the brother in our study had a normal delivery. Patient 1 and patient 2 were born at 37 weeks of gestation and patient 3 was born at 36 weeks of gestation, respectively. From their personal pathological medical history only patient 3 was discovered to have autoimmune thyroiditis when diagnosed with T1DM. The medical history characteristics of the patients are stated in Table 1. The clinical examination of the patients revealed no abnormalities.

Two 10 cc peripheral blood samples were obtained eight hours after the last meal for a blood chemistry and the HLA phenotyping. The later was made using an EDTA genomic DNA extraction kit compatible with Innupure C16 (Analytic Jena*96 tests/kit) and NGS Hybrid Capture (Next Generation Sequencing) AlloSeq Tx17 CareDx kit, using s.Illumina MiniSeq sequencer.

The microbiota analysis was performed from stool samples that were gathered during hospitalization or at home using a standardized procedure that involved antiseptic handling, collection in sterile tubes (without culture media), immediate freezing at −20 °C and DNA extraction using the PureLink Microbiome Purification Kit (Invitrogen). qRT-PCR was employed to determine the relative abundance of intestinal microorganisms in stool DNA isolated from both the patients and healthy controls (gender, age and environmentally matched), using a ViiA7© Fast Real-Time instrument (Applied Biosystems). Bacterial or fungal group-specific primers (16S rRNA and 18S rRNA, respectively) were utilized at their designated annealing temperatures and negative controls were included, consisting of samples without DNA templates. Universal 16S rRNA and 18S rRNA primers were used for normalization and relative abundance of bacterial abundance was calculated using the 2-ΔΔCt method.

The blood biochemistry showed inflammation in patient 2F and essential dyslipidemia in patient 2. Two of the three families (the family with siblings and the family in which the father suffers from T1DM) manifest high fasting glycaemia (patient 3B had 292 mg/dl, patient 3 had 370 mg/dl, patient 2F had 214 mg/dl and patient 2 had 287 mg/dl) although the values for Hb1c were in target for age. For all those involved in the study, the C-peptide at admission had a median of 1.5755 ng/ml +/- 0.85.

Table 1. Personal medical history of the patients.

Patient	1F	1	3B	3	2F	2
Gender	M	F	M	F	M	F
Age at onset	53	9	3	10	16	8
Current age	54	9	24	10	43	8
Gestational age	39	37	40	36	39	37
Weight at birth (g)		3000	3700	4500		2700
Length at birth (cm)		52	53	51		49
APGAR SCORE		9	9	9		9
Ketoacidosis at onset	0	1	1	1	0	1
Onset glycaemia (mg/dl)	365	320	450	300	420	212
Onset HbA1c	12.2	13	13.7	14	14.1	7

HbA1c= glycated hemoglobin.
1 - T1DM, 9-year-old female; 1F - the T1DM father of patient 1; 2 - T1DM, 8-year-old female; 2F - the T1DM father of patient 2; 3 - T1DM, 10-year-old female; 3B - the T1DM brother of patient 3.

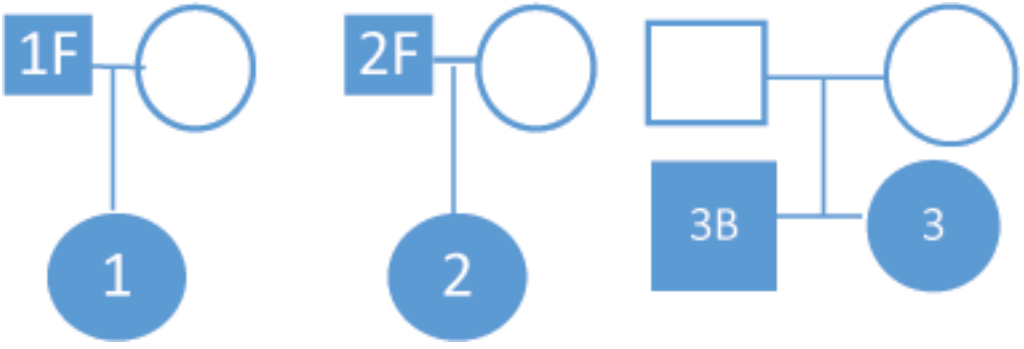

Fig. 1. The genetic penetrance of F1DM in the 3 cases (1-T1DM, 9-year-old female; 1F- the T1DM father of patient 1; 2- T1DM, 8-year-old female; 2F - the T1DM father of patient 2; 3- T1DM, 10-year-old female; 3B- the T1DM brother of patient 3).

The evaluation of other autoimmune diseases has revealed positive anti-thyroglobulin antibodies in all three families and positive ATPO antibodies in patient 1F, patient 3, patient 2F, but with negative antibodies for celiac disease (Table 2).

The thyroid function was normal except for patient 2, who had subclinical hypothyroidism with a TSH of 5.57 μUI /ml and a freeT4 of 1.31 ng/dl. The IGF-1 and the calcium-phosphate metabolism were normal, with insufficient levels of vitamin D in patient 3B and patient 3 (Table 3).

The HLA genetic evaluation has revealed that the haplotype DRB1*03:01-DQA1*05:01-DQB1*02:01 and DRB1*04:01/02/04/05/08-DQA1*03:01-DQB1*03:02/04 (or DR3/DR4) were present in all three families, but DPB1*02:02 and DPB1*03:01 which increase the risk are present in the paternal FT1DM families. Also, the highest risk heterogeneous genotype, HLADR3/4-DQ8 is found in the siblings with FT1DM, but also DPB1*04:02, which decreases the risk (Table 4).

The microbiota analysis of the stool samples from the three families has shown an abundance of *Enterobacteriaceae sp.* and *Candida sp.* and decreased levels of the protecting taxa *Akkermansia sp*. versus normal, clinically healthy age-matched subjects. In the case of the paternal FT1DM cases, a low abundance of *Bacteriodes sp.* was observed (Figure 2).

DISCUSSION

The Romanian FT1DM cases suggest a similar paternal predominance transmission, but with no maternal cases in the patients evaluated in a tertiary hospital. In the siblings with FT1DM the age gap is quite high, 14 years, but the studies cited in the literature indicate a small age gap between siblings, which increase the risk to FT1DM, as common childhood environmental factors may trigger the disease [16]. A more severe onset of disease in the three presented families has been observed, with diabetic ketoacidosis at presentation in all 6 cases, a high blood glucose and a high HbA1c [1,2].

The medical records of the patients indicate that most of them had a severe onset of the disease, accompanied by diabetic ketoacidosis, with a median onset glycaemia of 342.5 + /- 86.4 mg/dl and a median glycated hemoglobin (HbA1c) of 13.35.

Table 2. Autoimmune evaluation.

Patients	Patient 1F	Patient 1	Patient 3B	Patient 3	Patient 2F	Patient 2	Normal values
Anti-thyroglobulin Antibodies (IU/ml)	41.32	12.79	<10	50.34	253.9	10.38	1-16
ATPO (IU/ml)	28.8	<10	<10	385	104	<10	<20
Anti-transglutaminase Antibodies (IU/ml) -IgA	0.1	0.3	1.6	0.1	0.5	0.1	<10

ATPO=anti-thyroid peroxidase antibodies.
1 - T1DM, 9-year-old female; 1F - the T1DM father of patient 1; 2 - T1DM, 8-year-old female; 2F - the T1DM father of patient 2; 3 - T1DM, 10-year-old female; 3B - the T1DM brother of patient 3.

Table 3. Hormonal and calcium- phosphorous evaluation.

Patients	Patient 1F	Patient 1	Patient 3B	Patient 3	Patient 2F	Patient 2	Normal values
TSH (μUI/ml)	0.714	1.59	2.62	1.2	2.85	5.57	0.3-3.6
Free T4 (ng/dl)	0.904	1.45	1.25	0.96	1.08	1.31	0.8-1.48
25OH vitamin D (ng/ml)	39.6	51.58	20.04	22.43	34.33	58.53	>30
Total Calcium (mg/dl)	8.6	9	8.9	8.5	9.2	9.8	8.4-10.2
Phosphorous (mg/dl)	3.6	5.8	4.2	4.6	2.4	4	2.5-4.5
PKA (U/L)	51	211	106	136	68	125	30-120
Total Proteins (g/dl)	6.9	7.2	6.8	7.1	7.3	7.9	6-8
PTH (pg/ml)			38.28	38.33	93.4	19.76	15-65
IGF1 (ng/ml)	141	126	164	258	103	123	78-348

TSH = thyroid stimulating hormone; PKA = alkaline phosphatase; PTH = parathyroid hormone; IGF-1 = insulin-like growth factor 1.
1 - T1DM, 9-year-old female; 1F - the T1DM father of patient 1; 2 - T1DM, 8-year-old female; 2F - the T1DM father of patient 2; 3 - T1DM, 10-year-old female; 3B - the T1DM brother of patient 3.

Table 4. HLA characteristics of the patients.

Patients	Patient 1F	Patient 1	Patient 3B	Patient 3	Patient 2F	Patient 2
HLA-A	01:01:01 + 03:01:01	01:01:01 + 02:01:01	32:01:01 + 66:01:01	11:01:01 + 66:01:01	02:01:01 + 32:01:01	02:01:01 + 03:01:01
HLA-B	08:01:01 + 44:03:01	8:01:01	15:01:01 + 41:01:01	35:03:01 + 41:01:01	08:01:01 + 44:02:01	08:01:01 + 35:01:01
HLA-C	07:01:01 + 16:01:01	7:01:01	03:03:01 + 17:38:01	12:03:01 + 17:38:01	05:01:01 + 07:01:01	04:01:01 + 07:01:01
HLA-DPA1	1:03:01	01:03:01 + 02:01:02	1:03:01	01:03:01 + 01:04:01	01:03:01 + 01:04:01	1:03:01
HLA-DPB1	03:01:01 + 04:02:01	01:01:01 + 03:01:01	04:01:01 + 06:01:01	04:01:01 + 15:01:01	02:01:02 + 15:01:01	2:01:02
HLA-DQA1	02:01:01 + 05:01:01	5:01:01	03:01:01 + 03:03:01	3:03:01	01:03:01 + 05:01:01	01:01:01 + 05:01:01
HLA-DQB1	02:01:01 + 02:02:01	2:01:01	02:02:01 + 03:02:01	02:02:01 + 03:04:01	02:01:01 + 06:03:01	02:01:01 + 05:01:01
HLA-DRB1	3:01:01	3:01:01	04:04:01 + 04:05:01	04:05:01 + 04:08:01	03:01:01 + 13:01:01	3:01:01

1 - T1DM, 9-year-old female; 1F - the T1DM father of patient 1; 2 - T1DM, 8-year-old female; 2F - the T1DM father of patient 2; 3 - T1DM, 10-year-old female; 3B - the T1DM brother of patient 3.

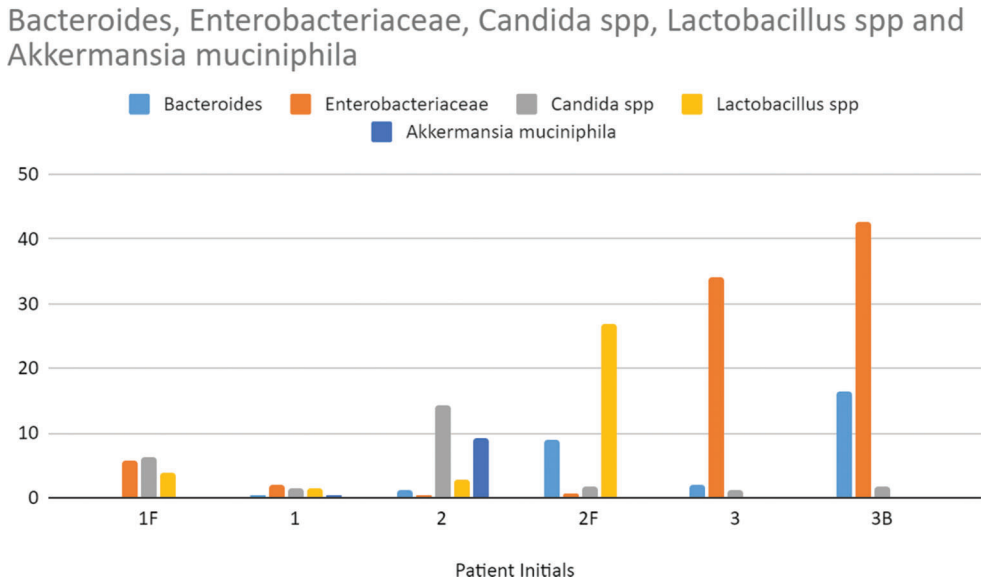

Fig. 2. Microbial relative abundance of the patient's gut microbiota.

The high onset HbA1c in two of the three girls (patient 1 and patient 3), even higher than in the case of their father/brother, might be explained in the paternal FT1DM case, by the relatively short period of time between the diagnosis of the father (a year) prior to the diagnosis of the daughter. This is also cited in a Finnish study that found that children with parents diagnosed with T1DM prior to birth have lower HbA1c at diagnosis, versus those whose parents were diagnosed after their birth [3].

No T1DM complications were observed in the six patients, and normal biochemistry was noticed. The patients were investigated for other autoimmune diseases, and thyroid autoimmunity has been observed in all 6 patients, with normal thyroid function, versus 21% of the spontaneous T1DM Romanian cases associated to autoimmune thyroid diseases cited in the literature [17].

Even though autoimmune thyroid disease was diagnosed in all 6 cases, celiac disease was not found, even though the allele DRB1*04-DQA1*03-DQB1*03:02 (known as DR4-DQ8) is associated with celiac disease [18].

The genetic evaluation of the patients shows the presence of the more frequent HLA DR3/DR4 in all 6 patients, with the same HLA-B, HLA-DPB1, HLA-DQA1, HLA-DQB1, HLA-DRB1for each family.

The HLA genetic evaluation revealed that the haplotype DRB1*03:01-DQA1*05:01-DQB1*02:01 and DRB1*04:01/02/04/05/08-DQA1*03:01-DQB1*03:02/04 (or DR3-DQ2 and DR4-DQ8) were present in all three families, with the haplotype DPB1*02:02 and DPB1*03:01, which increase the risk, found only in the paternal FT1DM families [8].

A heterogeneous genotype, HLADR3/4-DQ8, which has been found to trigger pancreatic autoimmunity before the age of 5, and which increases the prevalence of FT1DM, is found in the siblings with FT1DM, and not in the paternal FT1DM cases, even though fathers are believed to be responsible for transmitting the disease [4,19].

The DPB1*04:02 allele, which decreases the risk, is found just in the case of the siblings with FT1DM. However, the DRB1*04:05 allele, which has the highest risk of disease onset, and which is more potent than DPB1*04:02 allele, has also been found in the siblings. The protecting allele DRB1*04:03 is not found in any of the 6 cases [4,19].

Apart from the genetic predisposition of this form of type 1 diabetes mellitus, gut dysbiosis seem to be involved, characterized by the presence of pro-inflammatory bacteria – *Enterobacteriaceae* and *Candida Albicans*, and by diminished levels of protecting taxa- *Akkermansia muciniphila*, as found in other pediatric studies. The relative abundance of the gastrointestinal microbiota is similar between members of the same family, but different between families. Even if the pro-inflammatory taxa differ, all 6 patients have a higher risk dysbiosis profile than healthy age and environmental matched controls [10,14,20].

CONCLUSION

The present case series is the first one conducted in Romanian T1DM patients, with an evaluation of biological, hormonal, autoimmune, genetic, and gut microbiota determination that shows the same high paternal transmission, a more severe form, and a younger age at onset, but also a gut dysbiosis as other studies have found in the literature. The genetic evaluation supports these findings with the presence of predisposing allele that correlate with T1DM.

In conclusion, the analysis of gut dysbiosis and genetic evaluation has provided significant insights into familial type 1 diabetes mellitus in Romania. Regarding intestinal dysbiosis, an association with the presence of pro-inflammatory bacteria was identified, such as *Enterobacteriaceae* and *Candida Albicans*, alongside reduced levels of protective taxa, such as *Akkermansia muciniphila*, simiar to findings from other pediatric studies. Additionally, the dysbiosis profile differs between the examined families, although members of the same family tend to have similar intestinal microbial profiles. Concerning genetic evaluation, a significant association between certain predisposing alleles and FT1DM was identified. Families with FT1DM predominantly exhibited the haplotype DR3-DQ2 and DR4-DQ8, associated with an increased disease risk. Furthermore, certain alleles, such as DPB1*04:02 and DRB1*04:05, present in siblings with

FT1DM, may further influence the disease onset risk. In contrast, protective alleles, such as DRB1*04:03, were not identified in any of the six patients. These findings underscore the importance of careful screening of the intestinal microbiome profile and genetic analysis in identifying predisposing factors and the pathogenetic mechanisms involved in familial type 1 diabetes mellitus.

This case series may contribute to the risk detection of T1DM onset in the family members of patients; future studies with a higher number of patients and a longitudinal case control study aiming of delaying the disease onset by modulating the autoimmunity of these patients with pro and prebiotics are needed.

Informed consent

Written informed consent was obtained from the patients and their families, as part of the study conducted at the Pediatric Endocrinology and Diabetes Department of the Elias Emergency and University Hospital, in Bucharest, between 2019-2021 (protocol code 1695, 12.03.2019). All participants and all the planning, collecting, and reporting of the human data were in accordance with the Helsinki Declaration of Human Rights 2013, with the approval by the Ethics Commission for Scientific Research.

Conflict of interest

The authors declare that they have no competing interests.

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Nothing to declare.

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