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Role of fructosamine-3-kinase in protecting against the onset of microvascular and macrovascular complications in patients with T2DM

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ABSTRACT

Introduction Microangiopathic and macroangiopathic complications are the main cause of morbidity and mortality in the diabetic population. Numerous publications have highlighted the role of glycation in the onset of complications of diabetes. In this context, the detection of fructosamine-3-kinase (FN3K)—an enzyme capable of counteracting the effect of hyperglycemia by intervening in protein glycation—has attracted great interest. Several studies have linked *FN3K* genetic variability to its enzymatic activity and glycated hemoglobin (HbA1c) levels. Here, we investigated the role of *FN3K* polymorphisms in the development of microvascular and macrovascular complications of diabetes.

Research design and methods The anthropometric and biochemical parameters, and any medical history of microangiopathic and macroangiopathic complications, were documented in a sample of 80 subjects with type 2 diabetes. All subjects were screened for *FN3K* gene and analyzed for the combination of three polymorphisms known to be associated with its enzymatic activity (rs3859206 and rs2256339 in the promoter region and rs1056534 in exon 6).

Results The combination of allelic variants of *FN3K* polymorphisms resulted in 13 distinct genotypic variants within the cohort. Comparison between genotypes showed no significant differences in terms of demographic, anthropometric and biochemical parameters, risk markers and long-term complications, except for a higher age and vitamin E levels associated with the genotype presenting GG at position −385, TT at position −232, and CC at c.900 A. Evaluating the microangiopathic and macroangiopathic complications as a whole, we found that they appeared significantly less present in this genotype compared with all other genotypes (p=0.0306).

Conclusions The group of patients carrying the favorable allele for the three polymorphisms of the *FN3K* gene revealed less severe microangiopathy and macroangiopathy, suggesting a protective role of this genotype against the onset of the complications of diabetes.

Significance of this study

What is already known about this subject?

- Fructosamine-3-kinase (FN3K) is an intracellular enzyme involved in deglycation, active in removing ketoamines and preventing advanced glycation end product production.
- Some polymorphisms of the *FN3K* gene are associated with variations in HbA1c levels and with the onset of type 2 diabetes mellitus (T2DM) and pathogenic mechanisms related to its complications.

What are the new findings?

- Allelic variants of *FN3K* polymorphisms revealed 13 distinct genotypic variants in a cohort of patients with T2DM.
- Comparison between genotypes indicated similar demographic, anthropometric and biochemical parameters, risk markers and long-term complications, except for a higher age and vitamin E levels associated with the genotype presenting GG at position −385, TT at position −232, and CC at c.900 A.
- The *FN3K* genotype presenting GG at position −385, TT at position −232, and CC at c.900 A, is associated with less severe microangiopathic and macroangiopathic complications as a whole, compared to all other genotypes.

How might these results change the focus of research or clinical practice?

- The results suggest the opportunity of focusing research on *FN3K* genetic variability and on its potential applicability to the prevention, diagnosis and treatment of diabetes and its complications.

INTRODUCTION

Diabetes mellitus is a metabolic disorder that has become a public health problem because of its pandemic expansion worldwide.[1] The global prevalence of diabetes in 2019 was estimated at 9.3% (amounting to 463 million people), and it is expected to rise to 10.2% (578 million people) by 2030.[2] Diabetes is a complex heterogeneous disease in which genetic and environmental factors interact, causing hyperglycemia. Affected individuals are at high risk of morbidity and mortality due to the onset of microvascular and macrovascular complications.[3] Many hypotheses have

been advanced to explain the link between high blood sugar levels and the development of related complications.[4–6] Non-enzymatic protein glycation is etiologically linked to diabetic complications[6] and has been proven relevant in the diagnosis and treatment of diabetes, and the monitoring of a treatment's efficacy using glycated proteins such as HbA1c[7] or fructosamine.[8] Protein glycation is a common and spontaneous reaction dictated by blood glucose concentrations. It involves the binding of glucose or other reducing sugars to proteins, which leads to the formation of a broad and heterogeneous group of complex compounds called advanced glycation end products (AGEs).[9] While the generation of Schiff base and Amadori-rearranged ketoamine products is reversible in normal reaction kinetics,[10] there is another pathway for removing ketoamines and preventing AGE production catalyzed by fructosamine-3-kinase (FN3K).[11 12] FN3K is an intracellular enzyme found expressed in tissues most prone to glycation, such as the heart, nerves and kidneys.[13] Definitive evidence of FN3K involvement in deglycation has come from animal models, in which FN3K-knockout mice showed an increased protein glycation.[14] In diabetic subjects, single-nucleotide polymorphisms were found to alter FN3K activity, affecting HbA1c levels, the onset of type 2 diabetes mellitus (T2DM), and pathogenic mechanisms related to its complications.[13 15] Hence, there is great interest in the identification of an enzyme, FN3K, as part of a protein repair system for opposing the consequences of hyperglycemia.

Table 1 Anthropometric, clinical and biochemical characteristics of the 80 patients with T2DM

Parameters (unit of measure)	
Age (years)	69±8
Gender, M/F	41/39 (51%)
Waist circumference (cm)	102.4±10.9
BMI (kg/m^2)	30.3±5.3
Diastolic blood pressure (mm Hg)	79±11
Systolic blood pressure (mm Hg)	139±14
HbA1c (mmol/mol)	54.8±12.8
HbA1c (%)	7.2±1.2
Total cholesterol (mg/dL)	159±47
HDL cholesterol (mg/dL)	46±6
Triglycerides (mg/dL)	147±106
Microalbuminuria, yes/no	24/56 (30%/70%)
Retinopathy, yes/no	22/58 (27.5%/72.5%)
Cerebral macroangiopathy, yes/no	50/30 (62.5%/37.5%)
Peripheral artery disease, yes/no	11/69 (13.8%/86.2%)
Coronary heart disease, yes/no	4/76 (5%/95%)

Continuous variables are expressed as mean±SD. For frequency data, absolute values are given, with percentages in parentheses. BMI, body mass index; F, female; HDL, high-density lipoprotein; M, male; T2DM, type 2 diabetes mellitus.

In this study, we investigated the relationship between polymorphisms of the *FN3K* gene involved in the deglycation system (*FN3K* rs3859206, rs2256339 and rs1056534), and the onset of microvascular and macrovascular complications in individuals with T2DM.

RESEARCH DESIGN AND METHODS

Patients

Eighty consecutive patients with T2DM attending the outpatient Diabetes Center of the Department of Medicine at the University of Padova (Italy) were recruited. The mean age was 69±8 years (range 48–85 years, median 69 years), and the mean duration of diabetes was 14.6±4.3 years (range 8–20 years, median 15.5 years). T2DM was diagnosed in accordance with the WHO criteria,[16 17] which require fasting plasma glucose higher than 126 mg/dL. The sample's mean HbA1c levels were above the reference threshold (4%–6% in healthy people)[18] and were associated with the presence of complications of diabetes. As inclusion criteria, we considered a stable glycemic control demonstrated by two HbA1c measurements in the 6 months prior to enrollment, with a variation not exceeding 1%. Our exclusion criteria concerned all conditions that might cause changes in glycemia, such as disorders requiring steroid therapies or pregnancy, and any other conditions potentially affecting HbA1c concentrations (anemia, high erythrocyte turnover, bleeding, transfusions, hemoglobinopathies, liver and kidney diseases, and infections). The subjects included in the study were taking various treatments: 6 were on insulin therapy; 59 were on oral hypoglycemic therapy; and 15 were on both. During the clinical examination, the subjects' personal details (age and sex) and anthropometric data (BMI and waist circumference) were recorded, and their arterial blood pressure was measured. Peripheral venous blood samples were collected for biochemical and genetic analyses. For each subject, the following biochemical parameters were measured, using standard methods described elsewhere[19]: HbA1c, total cholesterol, high-density lipoprotein cholesterol, triglycerides, homocysteine, vitamin A and vitamin E.

Details of microangiopathic and macroangiopathic complications were obtained from the subjects' electronic medical records. Five parameters characterizing the microangiopathic and macroangiopathic complications typically associated with diabetes were examined: (1) microalbuminuria, calculated as the urine albumin:creatinine ratio (ACR), and classified as absent or present based on a cut-off of 30 mg/g; (2) diabetic retinopathy, detected on examination of the fundus oculi and classified as absent or present; (3) cerebral macroangiopathy, investigated by echo color Doppler study of the supra-aortic vessels and classified as absent or present; (4) peripheral artery disease, detected by measuring the ankle/arm index (ABI index) and classified as absent or present based on a cut-off of 0.9; and (5) coronary heart disease, assessed on the basis of clinical documentation

Table 2 Genotypes and allele frequencies of *FN3K* genetic variants identified in patients with T2DM

Promoter region variants		T2DM (n=80)	Exon/intron variants		T2DM (n=80)
c.-385 A>G (rs3859206)	AA	0.31	c.2 T>A	TT	0.99
	AG	0.54		AT	0.01
	GG	0.15		AA	/
	G-allele	0.42		A-allele	0.01
c.-232 A>T (rs 2256339)	AA	0.27	c.187 A>C (rs2253149)	AA	/
	AT	0.58		AC	/
	TT	0.15		CC	1
	T-allele	0.44		C-allele	0.01
c.-421 C>T	CC	0.99	IVS2-27 A>T	AA	0.99
	CT	0.01		AT	0.01
	TT	/		TT	/
	T-allele	0.01		T-allele	0.01
c.-429delATCGGAG	+/+	0.99	IVS2 +26 A>G (rs2253132)	AA	1
	+/del	0.01		AG	/
	del/del	/		GG	/
	del	0.01		A-allele	0.01
			IVS2 +31 A>T (rs2253131)	AA	/
				AT	0.19
				TT	0.81
				A-allele	0.09
			c.465 G>A	GG	0.99
				AG	0.01
				AA	/
				A-allele	0.01
			IVS4-9delTTG (rs72318398)	+/+	0.74
				+/del	0.25
				del/del	0.01
				del	0.14
			c.900 C>G (rs1056534)	CC	0.15
				GC	0.48
				GG	0.37
				C-allele	0.39
			c.906 C>T (rs149413139)	CC	0.98
				CT	0.03
				TT	/
				T-allele	0.01

Values for genotypes and rare alleles are frequencies.
rs ID: http://www.ncbi.nlm.nih.gov/snp/.
'+' symbol indicates the wild-type allele; 'del' denotes the allele characterized by the deletion.
FN3K, fructosamine-3-kinase; rs, RefSNP; T2DM, type 2 diabetes mellitus.

(clinical records, ECG, and echocardiograms) and classified as absent or present.

Sample size estimation of this exploratory study was based on an empirical assessment due to lack of knowledge of the frequency or values of parameters investigated.

Molecular analysis

DNA was extracted from subjects' whole blood samples using the QIAamp DNABlood Mini Kit (QIAGEN, Hilden, Germany). The procedure consists of four steps and was automated using the QIAcube (QIAGEN), a tool capable of processing QIAGEN balusters that enables

Table 3 Genotypes and their relative frequencies in the patients with T2DM investigated

Genotypes	c.-385A>G (rs3859206)	c.-232A>T (rs2256339)	c.900C>G (rs1056534)	T2DM (n=80)
A	GG	TT	CC	*0.1000*
B	GA	TA	CG	*0.2875*
C	AA	AA	GG	*0.1375*
D	AA	TA	CG	0.0375
E	AA	TA	GG	*0.1000*
F	GG	TA	CG	0.0500
G	GA	AA	GG	0.0750
H	GA	TA	GG	0.0500
I	GA	TA	CC	0.0500
J	AA	AA	CG	0.0250
K	AA	TT	GG	0.0125
L	GA	TT	CG	0.0375
M	GA	AA	CG	0.0375

Values for genotypes are frequencies. Frequencies ≥0.1 are in italics.
rs ID: http://www.ncbi.nlm.nih.gov/snp/.
rs, RefSNP; T2DM, type 2 diabetes mellitus.

automated low-yield sample preparation. Each whole blood sample was thawed and 200 µL was used for DNA extraction to obtain 3–12 µg of DNA, which was then amplified by PCR. Primers were designed to amplify the promoter region and the six exons, and corresponding intron/exon boundaries of the *FN3K* gene[20] in order to avoid amplifying any pseudogene located on chromosome 22.[21] Protocols specifically developed at our laboratory were used to amplify the largest number of exons with the same protocol. Because of its length, exon 6 was divided into two parts and amplified with two different PCR reactions. Online supplementary table 1 shows the length of the PCR products and some characteristics of the primers (sequence and melting temperature) used in each PCR amplification. The amplification protocol was completed using Biomek NXP (Beckman Coulter). The PCR products were then purified using the AMPure PCR Purification Kit (Agencourt Bioscience Corporation), automating the process with the Biomek 3000 (Beckman Coulter). Purified amplicons of the *FN3K* gene were then sequenced on both strands. The reaction plate with the sequencing mix was again automated using the Biomek 3000 (Beckman Coulter). Sequencing reaction products were purified using CleanSEQ Sequencing Reaction Clean-Up through the Biomek 3000 (Beckman Coulter). Finally, samples containing the sequencing reactions were loaded on a 48-capillary 3730 DNA Analyzer (Applied Biosystem), and the raw data were analyzed with the Sequencing Analysis software.

Statistical analysis

Data were expressed as mean±SD. The statistical analysis was performed using JMP13 software for Windows (SAS Institute, Cary, NC, USA). Student's *t*-test for independent samples was used to compare the data relating to the anthropometric and biohumoral parameters, as well as risk markers. The total complications were analyzed with the non-parametric Kruskal-Wallis test, followed by post hoc analysis. The χ^2 test was used to compare qualitative data. A p value of <0.05 was considered statistically significant.

The allelic frequencies for each polymorphism were calculated. The Heardy-Weinberg equilibrium (HWE) of the polymorphisms identified was estimated using the χ^2 test. The statistical analysis of our sample indicated that the HWE was respected for all the polymorphisms detected.

RESULTS

Table 1 shows the anthropometric, clinical and biochemical characteristics of the 80 subjects with T2DM enrolled in the present study, and the overall distribution of their microangiopathic and macroangiopathic complications.

All subjects were tested for the *FN3K* gene, considering all six exons with the corresponding intron–exon boundaries, and the promoter region. This screening identified 13 variants within the *FN3K* gene, 3 of which (c.2T>A, IVS2-27A>G and c.465G>A) were never previously reported. In addition, one of our subjects with T2DM revealed two variants previously identified in a single patient with type 1 diabetes mellitus (c.-421C>T and c.-429delATCGGAG)[22] (see table 2).

Referring to the literature, we sought genotypes comprising polymorphisms most associated with a variation in the enzymatic activity of FN3K in erythrocytes[23] in combination with a variation in HbA1c values.[13] The polymorphisms concerned are rs3859206, rs2256339 (in the promoter region), and rs1056534 (in exon 6) (table 2). The thirteen genotypes identified, labeled A to M, are listed in table 3. The genotypes GG at position −385, TT at position −232, and CC at c.900 have been associated with a better performance of the FN3K enzymatic activity.[23] The last one has been also associated with lower HbA1c levels.[13]

Only four of the genotypes (A, B, C, and E) had a frequency ≥0.1, so the others were grouped together. The clinical data collected for the study (population, anthropometric and hematochemical parameters, markers of risk, and microangiopathic and macroangiopathic complications) were associated with the five resulting genotype groups (A, B, C, E and Others) (table 4). The comparison between genotype groups revealed no statistically significant differences for any of the parameters considered, apart from age, glomerular filtration rate and vitamin E levels. In particular, genotype A was associated with a significantly older age than genotype C (p=0.0050) or the Others (p=0.0252), and genotype C was associated with a significantly younger age than genotype E (p=0.0445). Moreover, genotype A was associated with significantly higher vitamin E levels than genotype B (p=0.0365).

Table 4 Subjects' clinical characteristics by *FN3K* genotype group; for continuous data, values are mean±SD

Parameter	A genotype	B genotype	C genotype	E genotype	Others
n	8	23	11	8	30
Age (years)	74±8[C,Others]	69±8	64±7[E]	71±7	68±7
Gender, M/F	4/4	8/15	7/4	5/3	17/13
Diabetes duration (years)	12.4±4.0	14.6±4.5	15.7±3.9	13.1±4.6	15.1±4.2
BMI (kg/m^2)	31±5	30±6	31±5	29±4	30±5
Waist circumference (cm)	102±8	103±12	105±13	100±7	101±11
Systolic blood pressure (mm Hg)	135±12	135±12	138±13	139±12	137±10
Diastolic blood pressure (mm Hg)	79±6	80±9	79±7	80±10	81±7
DCCT-HbA1c (%)	7.2±0.7	7.6±1.0	7.3±0.5	7.7±1.1	7.2±0.7
IFCC-HbA1c (mmol/mol)	54.8±7.6	59.3±11.3	56.0±6.2	59.6±14.0	54.9±6.9
Total cholesterol (mg/dL)	181±33	180±48	158±28	188±23	179±32
HDL cholesterol (mg/dL)	56±20	57±18	55±10	47±9	50±14
Triglycerides (mg/dL)	131±57	105±47	96±36	131±41	128±61
Microalbuminuria, yes/no	8/0	3/20	3/8	3/5	7/23
Albuminuria (mg/g)	5±4	31±65	22±21	19±19	28±34
Creatininemia (mg/dL)	0.9±0.3	0.8±0.2	0.9±0.2	1.0±0.2	0.8±0.2
Glomerular filtration rate (mL/min)	77±16	84±20	82±17	71±14	89±21[E]
Homocysteine (µmol/L)	17±3	16±5	18±8	18±7	15±5
Vitamin A (µg/dL)	56±15	51±15	45±10	52±10	52±17
Vitamin E (mg/dL)	1.5±0.3[B]	1.3±0.3	1.3±0.3	1.3±0.2	1.3±0.3
Cerebral macroangiopathy, yes/no	4/4	16/7	5/6	5/3	20/10
Coronary heart disease, yes/no	0/8	3/20	1/10	0/8	0/30
Retinopathy, yes/no	0/8	5/18	5/6	1/7	11/19
Peripheral artery disease, yes/no	0/8	3/20	1/10	2/8	5/25

Superscript letters indicate significant differences between respective genotype groups.
BMI, body mass index; DCCT-HbA1c, HbA1c according to Diabetes Control and Complications Trial units; F, female; FN3K, fructosamine-3-kinase; HDL, high-density lipoprotein; IFCC-HbA1c, HbA1c according to International Federation of Clinical Chemistry units; M, male.

Microangiopathic and macroangiopathic complications were considered together, and their frequency was compared between the different genotype groups (figure 1). The non-parametric Kruskal-Wallis test indicated a significantly different number of complications among the groups (p=0.0306). Following a post hoc analysis, a significant difference emerged between genotype A and genotypes B (p=0.0145), C (p=0.0273), E (p=0.0373), and the Others (p=0.0024).

DISCUSSION

Recent years have seen a growing role of precision medicine in efforts to identify prevention, diagnosis and treatment strategies targeting particular categories of patients, stratifying populations by applying genomics to develop individual phenotypic profiles of disease.[24] In the field of diabetes, one of the currently most intriguing fields of investigation focuses on glycation. Several studies have shown that glycation is associated with a condition of chronic hyperglycemia and the consequent development of long-term complications of diabetes.[25] In this setting, the identification of FN3K (an enzyme capable of preventing the effects of hyperglycemia by intervening on protein glycation, and thereby on the damage mechanisms responsible for the onset of diabetic complications) has aroused great interest.[26]

In this exploratory study, the *FN3K* gene was analyzed by direct sequencing in a cohort of 80 patients with T2DM. Three polymorphisms within the *FN3K* gene, found relevant in the literature,[13 22 23 27] were taken into consideration: c.-385A>G (rs3859206) and c.-232A>T (rs2256339), located in the promoter region, and c.900C>G (rs1056534), located on exon 6 (table 2). Thirteen genotypes were identified (table 3), and clinical data were compared by genotype (table 4).

No significant differences in subjects' demographic, anthropometric or biohumoral parameters emerged between the different genotype groups (with the exceptions of older age and higher vitamin E levels for the genotype A, and glomerular filtration rate between genotype E and Others). A similar trend was seen for the risk factors investigated.

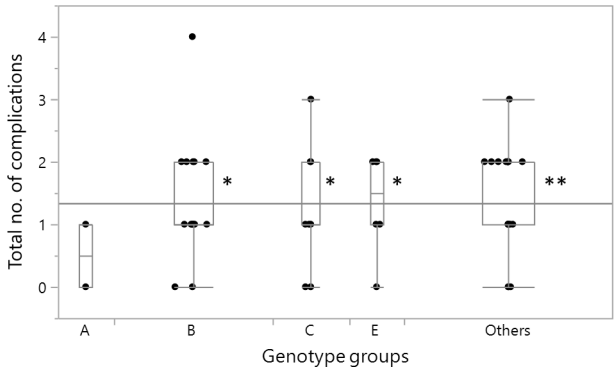

Figure 1 Box plot showing the distribution of the combined microangiopathic and macroangiopathic complications in the *FN3K* genotype groups. The total number of complications per patient includes the presence of at least one of the following: cerebral macroangiopathy, peripheral artery disease, ischemic heart disease, retinopathy, and microalbuminuria. The Kruskal-Wallis test indicated a statistically significant difference among the genotype groups (p=0.0306). **p<0.01, *p<0.05 post hoc analysis versus genotype group A. In the graph, the edges of the box indicate the 25th and 75th quantiles, including the middle 50% of the data; whiskers indicate the range of data, calculated as [upper quartile+1.5 (IQR)] and [lower quartile−1.5 (IQR)]; the continuous horizontal line is the overall arithmetical mean for all the data; the scattered points are single subject's values. The width of each box is proportional to the number of cases in each group. FN3K, fructosamine-3-kinase.

Intriguingly, when microangiopathic and macroangiopathic complications were pooled together, genotype A (deriving from the combination of the favorable alleles GG in c.-385A>G, TT in c.-232A>T, and CC in c.900C>G) showed a statistically significant inverse relationship with their occurrence. This might mean that genotype A could be a factor in preventing the onset of vascular complications. To the best of our knowledge, this finding is new and in line with previous studies on FN3K. In particular, Delpierre *et al*[23] reported that, when analyzed individually, the alleles in genotype A were associated with a better performance of the enzymatic activity of FN3K, coinciding with a lower level of glycation. Škrha *et al*[15] correlated the GG allele of the c.900C>G polymorphism with a greater production of soluble receptors for advanced glycation end products. Our study suggests that the CC allele also contributes to the protective effect associated with genotype A.

The severity of long-term diabetic complications is usually age-related.[28] Intriguingly, patients with genotype A and with a better outcome in terms of vascular complications are older than the patients belonging to the other genotypic groups. This observation indirectly reinforces our finding of a protective role of FN3K in the development of vascular complications of diabetes.

One of the limitations of our study concerns the small sample size of the genotype groups, which may explain the lack of significant differences between the various genotypes when microvascular and macrovascular complications were analyzed separately. However, the subdivision into genotype groups was not predictable a priori, and therefore the reduced number of subjects resulting from the initial T2DM cohort unfortunately caused a reduction of the study power. Despite this limitation, there was an evident difference in overall microangiopathic and macroangiopathic complications between genotype A and all the other genotypes. The genotype effect on individual complications, leading to non-significant results for the complications considered separately, would deserve further investigation, also aimed at evaluating the potentially protective effect of genotype A on FN3K activity in a larger cohort. In this light, the recent report from Dunmore *et al*[29] is highly relevant, as it demonstrates a relationship between the enzymatic activity of FN3K and the glycation gap. Compared with HbA1c levels, the glycation gap is a more reliable indicator of glyco-oxidative stress (and the consequent risk of developing complications), linked to predictions based on fructosamine levels.[8 30 31] It will be worth integrating the *FN3K* genetic variants with the glycation gap to shed more light on the possible protective role of genotype A that emerged from our study.

Another limitation of our study regarding the possible determination of the deglycating products of the *FN3K* variants we have genotyped. Indeed, we performed some experiments to measure the FN3K catalytic activity by adopting the procedure described by Krause *et al*,[32] based on the conversion of the synthetic UV-active fructosamine Nα-hippuryl-Nε-(1-deoxy-D-fructosyl)lysine (BzGFruK) to Nα-hippuryl-Nε-(phosphofructosyl)lysine (BzGpFruK). The substrate has been synthetized, but the phosphorylated substrate was very unstable and very difficult to be quantified in a reproducible way. A more promising and simple method was developed later by another group,[33] but we were not convinced of the substrate these authors were using (ie, bovine albumin glycated in vitro) because previous experience with this substrate also showed its marked instability. So, at least according to our experience, the determination of FN3K remains still an open issue.

The identification of a genotype with a protective role may open up new prospects for research on *FN3K* genetic variability and on its potential applicability to the prevention, diagnosis and treatment of diabetes and its complications.

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Contributors GS designed the study, interpreted the data and revised the manuscript. AM, RP and FA carried analyses and contributed to interpretation of data. ER analyzed and interpreted the data, and wrote the manuscript. NCC and

SB provided patient samples and contributed to the discussion. AL reviewed and critically edited the manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

Ethics approval The study used information that is available in the database of the Department of Medicine (DIMED), University of Padua. The study was approved by ethical committee for clinical trials in Padua (approval no.149 DCCT/HBA1C) and was conducted in accordance with the Helsinki Declaration. Participants gave informed consent before taking part to the study.

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Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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