Universidade de Lisboa

Faculdade de Farmácia

Molecular diagnosis of Maturity Onset Diabetes of the Young: a case for personalized medicine

Daniela Mendes Poejo

Dissertação orientada pela Doutora Mafalda Bourbon e coorientada pela Professora Doutora Cecília Rodrigues.

Mestrado em Ciências Biofarmacêuticas

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Resumo

A diabetes mellitus, ou apenas diabetes, como é usualmente conhecida, é um dos fatores de maior risco para o desenvolvimento de doenças cardiovasculares. Esta patologia é caracterizada por um conjunto de alterações metabólicas causadas pela deficiência na secreção da hormona insulina. A resposta ineficaz da insulina conduz a um estado de hiperglicemia permanente desencadeada pelo aumento dos níveis de glicose no sangue. Apesar da diabetes mellitus tipo 1 e a diabetes mellitus tipo 2 serem as mais frequentes e consequentemente mais estudadas, existem vários tipos de diabetes mellitus que diferem tanto nas características clínicas como na sua fisiopatologia. Desde modo, é essencial considerar as formas mais raras de diabetes adequando o tipo de intervenção terapêutica de acordo com as características específicas do tipo de diabetes e do paciente. A diabetes tipo MODY (*Maturity Onset Diabetes of the Young*) é uma forma monogénica de diabetes descrita pela primeira vez em 1974 por Tattersall. A diabetes tipo MODY é uma doença de origem genética de padrão de hereditariedade autossómico dominante que provoca alterações no normal funcionamento nas células responsáveis pela produção de insulina, as células beta do pâncreas. O aparecimento deste tipo de diabetes costuma manifestar-se em crianças e jovens antes dos 25 anos, em indivíduos não-insulino dependentes e sem sinais de insulinorresistência. Apesar de apresentar tanto características clínicas como base genética díspares, pacientes com diabetes tipo MODY são muitas vezes erradamente diagnosticados com diabetes tipo 1 ou diabetes tipo 2. Além disso, a diabetes tipo MODY apresenta uma elevada heterogeneidade fenotípica e genotípica fazendo com que o teste genético seja extremamente relevante tanto para a otimização do tratamento como para a prevenção de possíveis complicações de saúde associadas a diabetes.

Atualmente, sabe-se que alterações genéticas em 1 dos 14 genes associados à MODY podem ser causa patogénica da diabetes deste tipo. Mutações heterozigóticas nos genes *GCK*, *HNF1A*, *HNF4A* e *HNF1B* são as causas mais comuns encontradas em pacientes com diabetes tipo MODY.
Localizado no cromossoma 7 (7p13), o gene *GCK* codifica a enzima glucocinase (*GCK – glucokinase*) que desempenha um papel fundamental na primeira fase dos processos metabólicos da glicose. As isoformas desta enzima monomérica estão presentes no fígado e células beta do pâncreas. Nestas últimas, atuam como um sensor do nível de glicose induzindo a secreção de insulina. Quando ocorrem alterações patogénicas neste gene a atividade enzimática da GCK fica comprometida. A diminuição da atividade da GCK afeta diretamente a normal secreção de insulina, aumentando a concentração mínima de glicose no sangue necessária para a estimulação da libertação de insulina. Assim, indivíduos com diabetes tipo MODY, subtipo *GCK* apresentam hiperglicemia moderada desde a nascença, podendo ser em muitos casos assintomáticos.
O gene *HNF1A* está localizado no cromossoma 12 (12q24.31), codifica um fator de transcrição *hepatocyte nuclear factor 1 alpha* pertencente a um grupo de fatores de transcrição hepáticos que estão associados ao aparecimento a longo prazo de complicações de saúde derivadas da diabetes. Este fator de transcrição possui três isoformas A, B e C que estão distribuídas diferencialmente em vários orgãos, como fígado, rim e pâncreas. O seu perfil de expressão difere consoante o órgão e o estado de desenvolvimento do mesmo. Enquanto a isoforma A está presente em fases iniciais de desenvolvimento do pâncreas fetal, a isoforma B é predominante no pâncreas maturo. Além disso, sabe-se que *HNF1A* regula a proliferação e morte celular das células beta tendo um papel crucial na expressão de insulina.Variantes genéticas neste gene levam a uma desregulação na proliferação das células beta desencadeando uma deficiência progressiva na secreção de insulina. Os níveis de insulina endógena de pacientes com este

subtipo *HNF1A* de diabetes tipo MODY tendem a diminuir ao longo da vida. Desde modo, estes pacientes necessitam de um tratamento contínuo que deverá ser periodicamente ajustado. À semelhança do anterior, o gene *HNF1B* pertence ao mesmo grupo fatores de transcrição. Está localizado no cromossoma 17 (17q12) e codifica para o fator de transcrição *hepatocyte nuclear factor 1 beta*. O gene *HNF1B* é expresso em inúmeros tecidos como é o caso do timo, pulmão, estômago, intestino, fígado, rim, pâncreas e trato genital. Destacando a sua função em fase prematura do desenvolvimento das estruturas dos tecidos. De facto, *HNF1B* desempenha um papel essencial no desenvolvimento embrionário de vários órgãos, como é o caso do pâncreas e do rim. Alterações patogénicas no gene *HNF1B* são caracterizadas pela disfunção de células beta combinada com um uma diminuição da sensibilidade à insulina. Adicionalmente, é bastante comum em paciente com este subtipo de MODY o desenvolvimento de disfunções renais e complicações cardiovasculares. Assim, pacientes com este subtipo de diabetes tipo MODY precisam de um controlo glicémico adequado para prevenir e ou atenuar os problemas de saúde associados, onde a administração de insulina é frequentemente utilizada.

Neste estudo participaram dezanove indivíduos previamente selecionados com características clínicas que permitissem suspeitar de diabetes tipo MODY. As informações clínicas foram recolhidas através de um questionário enviado por uma médica da Associação Protetora dos Diabéticos de Portugal (APDP), que indicou estes pacientes para o estudo molecular de diabetes tipo MODY que decorre no Instituto Nacional de Saúde Dr. Ricardo Jorge. O DNA de cada participante foi extraído a partir das amostras de sangue colhidas aos participantes. Posteriormente os genes *GCK*, *HNF1A* e *HNF1B* foram amplificados por PCR e estudados utilizando o método de sequenciação de Sanger. Foram utilizadas ferramentas bioinformáticas para o alinhamento e análise de variantes nos cromatogramas obtidos. Por fim, as variantes encontradas foram classificadas segundo os critérios da *American College of Medical Genetics and Genomics*.

Como resultado da sequenciação de Sanger foram detetadas no total dezasseis variantes com distribuição não uniforme ao longo de dois genes estudados: três variantes no gene *GCK* e treze variantes no gene *HNF1A*. Sete destas variantes foram classificadas como de significado incerto. O número de doentes não relacionados com a variante, o tipo de variante e sua localização, a prevalência da variante em populações controlo, a co segregação da variante com o fenótipo na família e os fenótipos apresentados pelos participantes são algumas das evidências que permitiram interpretar e classificar estas variantes. Infelizmente, devido à falta de informação disponível, não foi possível atribuir uma classificação definitiva a estas 7 variantes. Assim, estas variantes foram classificadas como de significado incerto até que seja possível obter mais dados que suportem a revisão da sua interpretação.

Em resumo, foram encontradas sete variantes em sete participantes classificadas como variante de significado incerto e nos restantes doze indivíduos em estudo as variantes encontradas foram classificadas como benignas não estando associadas à patologia. É necessário continuar o estudo dos outros genes associados a MODY e aumentar a evidência sobre as variantes encontradas de forma a que estes doentes tenham um diagnóstico definitivo. Este estudo realça a importância da implementação do teste genético em pacientes diabéticos com fenótipo de MODY. Da mesma forma, o investimento na investigação de diabetes tipo MODY é essencial para a melhor interpretação e classificação de variantes. O correto diagnóstico e a identificação genotípica de indivíduos com diabetes tipo MODY permite melhor adequar as opções terapêuticas. A personalização do tratamento de acordo com as características genéticas de cada subtipo pode ser um enorme avanço na melhoria do controlo glicémico e na prevenção de outras complicações de saúde tendo grande impacto na qualidade de vida dos pacientes e famílias.

Palava-chaves:

Diabetes monogénica; MODY; *GCK*; *HNF1A*; *HNF1B*

Abstract

Diabetes mellitus, or simply diabetes how it is most known, is one of the major risk factors for cardiovascular disease. It is characterised by several metabolic disorders caused by a deficient segregation of the insulin hormone. An inadequate insulin response triggers chronic hyperglycaemia due to higher glucose levels in the blood. There are several different types of diabetes, according to pathophysiology. Despite type 1 and type 2 diabetes mellitus being the overwhelming majority of the cases, there are rare types of diabetes which demand a specific healthcare approach. Maturity Onset Diabetes of the Young (MODY) is an early onset monogenic diabetes firstly described by Tattersall in 1974. Even though present features and treatment requirements differ from other types of diabetes, it is commonly misdiagnosed as type 1 or type 2 diabetes. MODY is an autosomal dominant inherited disorder caused by β-cell dysfunction, characterised by a vast range of different phenotypes due to genetic heterogeneity. In fact, 14 genes have been associated with MODY phenotypes which reinforces the importance of genetic testing. Heterozygous pathogenic variants in the *GCK*, *HNF1A*, *HNF4A*, and *HNF1B* genes are the four most common causes of this type of diabetes.

Glucokinase (*GCK*) is a monomeric enzyme that plays an essential role in the first step of glucose metabolism, acting as a glucose sensor for insulin release from pancreatic β-cells. Heterozygous pathogenic variants might lead to a reduction of *GCK* enzymatic activity and consequent effects on normal insulin segregation. Therefore, GCK-MODY patients often present asymptomatic mild state hyperglycaemia with no oral hypoglycaemic agents (OHA) or insulin therapy needs. Hepatocyte nuclear factor 1 alpha (*HNF1A*) is a hepatocyte nuclear factor involved in cell cycle control and the regulation of several genes. Defects in *HNF1A* trigger impairment of β-cell proliferation and progressive insulin release deficiency. HNF1A-MODY patients usually require treatment over their lifetime since the endogenous insulin tends to be decreased These patients need special management for glycaemic control and other cardiovascular risk factors since they have an increased cardiovascular risk. In these cases, the hypoglycaemic agent that has a higher success rate is sulphonylurea. Similar to *HNF1A*, hepatocyte nuclear factor 1 beta (*HNF1B*) is also a hepatocyte nuclear factor that has an essential role in embryonic development in different tissues, including the pancreas and kidney. Pathogenic variants in *HNF1B* have been characterised by β-cells dysfunction combined with insulin resistance. Also, extra-pancreatic complications, such as renal dysfunctions and microvascular disease, are wide-spread and frequent. Due to insulin resistance, HNF1B-MODY patients require exogenous insulin administration. Genetic testing is essential to distinguish between types of diabetes, providing personalised and effective treatment for patients who present MODY clinical features. Thus, this project aimed at the genetic identification of MODY variants in patients with a MODY clinical diagnosis.

Nineteen participants have been referred to this study, which took place in National Health Institute Dr. Ricardo Jorge. Participants had been previously selected as clinical MODY patients. The inclusive criteria were early onset of diabetes, absence of pancreatic antibodies, no-signal of insulin resistance, and strong family history of any diabetes. Clinical data were collected from a questionnaire sent by a clinician from the Portuguese Diabetic Association. DNA from probands were extracted from whole blood in EDTA tubes. Next, the samples were studied by performing PCR and Sanger sequencing. Sequencing chromatograms were analyse using bioinformatic tools. Lastly, variants were classified following the American College of Medical Genetics and Genomics guidelines.

A total of sixteen variants were detected with a non-uniform distribution among two studied genes: three variants in *GCK* and thirteen in *HNF1A*. Among the detected variants, seven variants were classified as uncertain significance. The number of unrelated patients with the variant, the minor allele frequency, the type and localization of the variant, variant co-segregation in relatives with phenotype, and specific clinical features presented by patients and other specific criteria are the evidence needed to be collected for the interpretation and classification of variants. Due to lack of evidence towards pathogenicity seven variants did not have enough available information for more accurate classification and so were classified as variants of unknown significance until more data would be available.

Among 19 participants in this study, a total of seven variants of unknown significance were found in seven participants, and the remaining variants found in twelve participants were benign, not being associated with MODY. It is necessary to continue the study of the other genes associated with MODY and enlarge the evidence on the variants found in order to provide a definitive diagnosis to these patients. This study emphasises the importance of genetic testing for individuals with a clinical phenotype of MODY. Thus, a correct diagnosis and identification of the genetic etiology are important to adequate and personalise treatment. Customizing treatment according to the genetic information of each subtype can be a huge step forward in improving glycaemic control and preventing other health complications that have a major impact on the quality of patients and family's lifestyle.

Key words:

Monogenic diabetes; MODY; *GCK*; *HNF1A*; *HNF1B*

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Abbreviations, acronyms, and symbols

ABCC8	ATP Binding Cassette Subfamily C Member 8 (gene)
ACMG	American College of Medical Genetics and Genomics
APDP	Associação Protectora dos Diabéticos de Portugal
apoAII	Apolipoprotein A-II
apoB	Apolipoprotein B
apoCIII	Apolipoprotein C-III
APPL1	Adaptor Protein, Phosphotyrosine Interacting with PH Domain and Leucine Zipper 1 (gene)
ASD	Autism Spectrum Disorder
ATP	Adenosine Triphosphate
BLK	B Lymphocyte Kinase (gene)
BMI	Body Mass Index
CEL	Carboxyl Ester Lipase (gene)
CNV	Copy Number Variation
COSMIC	Catalogue of Somatic Mutations in Cancer
CVD	Cardiovascular disease
dbSNP	Single Nucleotide Polymorphism
del	Deletion
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside Triphosphate
EDTA	Ethylenediaminetetraacetic Acid
FPG	Fasting Plasma Glucose
G6P	Glucose-6-Phosphate
GADA	Glutamic Acid Decarboxylase Antibodies
GCK	Glucokinase (gene)
GLUT2	Glucose Transporter Molecule
gnomAD	Genome Aggregation Database
HbA1c	Glycated Haemoglobin
HDL	High-Density Lipoprotein
HGMD	Human Gene Mutation Database
HNF1A	Hepatocyte Nuclear Factor 1 Alfa (gene)
HNF1B	Hepatocyte Nuclear Factor 1 Beta (gene)
HNF4A	Hepatocyte Nuclear Factor 4 Alfa (gene)
ICA	Islet Cell Antibodies
INS	Insulin (gene)
ins	insertion
KCNJ11	Potassium Voltage-gated Channel Subfamily J Member 11 (gene)
KLF11	Kruppel Like Factor 11 (gene)
MAF	Minor Allele Frequency
MAF popmax	Minor Allele Frequecy in population with highest frequency

MAXENTSCAN	Maximum Entropy Scan
MLPA	Multiplex Ligation-dependent Probe Amplification
MODY	Maturity Onset of Diabetes of the Young
mRNA	Messenger RNA
NA	Not Applicable
NCBI	National Center for Biotechnology Information
NEUROD1	Neuronal Differentiation 1 (gene)
NF	Not Found
NGS	Next-Generation Sequencing
NNSLIPE	Splice Site Prediction by Neural Network
OGTT	Oral Glucose Tolerance Test
OHA	Oral Hypoglycaemic Agents
PAX4	Paired Box 4 (gene)
PCR	Polymerase Chain Reaction
PDX1	Pancreatic and Duodenal Homebox 1 (gene)
PolyPhen-2	Polymorphism Phenotyping v2
popmax	population with highest frequency
PROVEAN	Protein Variation Effect Analyzer
REVEL	Rare Exome Variant Ensemble Learner
RNA	Ribonucleic acid
SGLT-2	Sodium-Glucose Cotransporter 2
SIFT	Sorting Intolerant From Tolerant
SNP	Single-nucleotide polymorphism
SUR1	Sulfonylurea Type 1
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
Taq	Taq DNA polymerase
TBE	Tris/Borate/EDTA
UK	United Kingdom
UV	UltraViolet
β-cells	Beta Cells

Introduction

In the few last years, millions of cases of diabetes mellitus have been recorded all over the world. Unhealthy lifestyles and a global increase in obesity rates are critical factors that contribute to an increasing number of diabetic cases over the next decades. A change of lifestyle and a balanced diet may be a way to reverse the situation, avoiding more severe health complications. However, genetic factors can also be involved in the development of diabetes. Therefore, the knowledge about genetic information on diabetic patients is essential, as well as the understanding of the origin of the disease, which would open the possibility of personalized therapeutic approaches [1].

Diabetes Mellitus

Diabetes Mellitus is a group of chronic diseases resulting in metabolic disorders due to elevated glucose levels in the blood, which in long-term damage different organs [2]. Despite having several pathological processes associated with the development of diabetes, the normal activity of insulin is always compromised [2] [3]. Insulin is an essential hormone for controlling the levels of glucose in the blood. Moreover, it is involved in vasodilation and regulates lipid flux [4]. Insulin synthesis occurs mainly as a response to glucose, even though it may also respond to sulphonylureas and arginine [5]. Thus, diabetic patients are characterized by high sugar levels in the blood due to the deficiency or absence of insulin (hyperglycaemia) [3] [6]. Chronic hyperglycaemia may lead to several disorders in different organs, such as the nerves, the heart, and blood vessels [3]. Among the various cardiovascular complications, retinopathy can be highlighted, which in advanced stages can culminate in diabetic blindness, nephropathy that is associated with kidney problems, and neuropathy that is characterized by damages in the peripheral nerves and can affect different systems [2] [7].

Some evidence points out that diabetes is associated with other clinical conditions such as hypertension and lipoprotein metabolism abnormalities (dyslipidaemia), which is the main cause of atherosclerotic plaque development. All these factors contribute to the increased risk of cardiovascular disease (CVD) in diabetics [3]. In fact, myocardial infarction is the most common cause of death in people with diabetes, making diagnosing and treating diabetes essential to prevent the risk of cardiovascular events [7] [8].

Different diabetes types

The pathologic process of diabetes is much heterogeneous. The lack of bioavailability of insulin could be originated by damage of β-cells of the pancreas (cells responsible for insulin production) or by abnormalities that lead to insulin resistance [3] [9]. The cases of diabetes must be divided according to etiopathogenetic categories being the most common type 1 and type 2 diabetes [3]. Type 1 diabetes (T1DM) is characterized by the immune destruction of pancreatic β-cells leading to an absolute deficiency of insulin secretion. These patients have high autoimmune antibody concentrations, which is the main differential feature [2]. On the other hand, type 2 diabetes (T2DM) is caused by increased insulin resistance and inadequate insulin response. In T2DM cases, the later onset of symptoms is widespread, although, during the asymptomatic period, hyperglycaemia already has a negative effect causing damage to tissues. Despite the genetic predisposition still being unclear, environmental factors like age, obesity, and sedentary lifestyle, have an essential role in the onset of this type of diabetes [2]. Even though the previously mentioned types of diabetes are the most common, there are other

types of diabetes with different features, such as Maturity Onset Diabetes of the Young (MODY). MODY is usually misdiagnosed as type 1 or type 2 diabetes [9]. MODY will be the focus of this project.

MODY

Maturity Onset Diabetes of the Young (MODY) was characterized for the first time in 1974 by Tattersall [10] as a monogenic form of early-onset diabetes [11]. MODY has a prevalence of 1-2% of all cases of diabetes being the most common monogenic type of diabetes [9] [12]. Several genes may be responsible for MODY phenotypes. Genetic mutations in these genes lead to a β-cell dysfunction. The β-cells play an essential role in insulin segregation, producing proinsulin, which is cleaved, forming insulin and C peptide [9] [13]. MODY has an autosomal dominant pattern without signals of autoimmunity or insulin resistance, and is usually present in adolescence or young adulthood (typically age <25 years) [9] [12] [14] [15].

It is quite common to find different clinical features in patients with MODY, these different phenotypes are a consequence of genetic heterogeneity. Depending on the genetic etiology, the different genetic subtypes differ in terms of age of onset, pattern of hyperglycaemia, response to treatment, and extra-pancreatic manifestations [16]. However, most patients with this type of diabetes usually have low insulin requirement. They are often characterized by a strong family history of diabetes of any type, non-insulin dependence, absence of evidence of β-cell autoimmunity and the presence of C peptide which indicates the producing of endogenous insulin [11] [16]. Furthermore, in the case of running out of insulin, subjects with MODY rarely develop diabetic ketoacidosis. Despite all these standard features allowing suspicion of a MODY phenotype, genetic testing is needed to ensure a correct diagnosis and identify the related gene and variants, important to define the best therapeutic choice [16].

MODY Genes

Since the first clinical description, several molecular genetics advancements led to identification of pathogenic variants in some genes that are involved in the β-cell function. This knowledge allowed to recognize clinical phenotypes according to several distinct features as MODY phenotypes [11]. Nowadays, pathogenic variants in 14 genes are associated with MODY phenotypes [15]. According to each country's blood glucose monitoring practices, it is possible to detect some different gene frequencies even though it is consensual that pathogenic variants in glucokinase (*GCK*) and hepatocyte nuclear factors 1α, 4α and 1β (respectively *HNF1A*, *HNF4A*, and *HNF1B*) are the four most likely causes of MODY [9] [14] [15]. Additionally, lack of data for the remaining 10 genes representing only 20% of all pathogenic variants of MODY, compromises the significance of variants found and their pathogenicity [15]. The following table (Table 1) shows the different types, each gene MODY-associated, and the probability of occurring a pathogenic variant [15].

Table 1 Genes associated with Maturity Onset Diabetes of the Young (MODY) and respective frequencies.

Locus Name	Gene	Full name gene	% of all MODY
MODY 1	*HNF4A*	hepatocyte nuclear factor 4 alpha	5%-10%
MODY 2	*GCK*	glucokinase	30%-50%
MODY 3	*HNF1A*	hepatocyte nuclear factor 1 alpha	30%-65%
MODY 4	*PDX1*	pancreatic and duodenal homebox 1	1%
MODY 5	*HNF1B*	hepatocyte nuclear factor 1 beta	<5%
MODY 6	*NEUROD1*	neuronal differentiation 1	<1%
MODY 7	*KLF11*	Kruppel like factor 11	<1%
MODY 8	*CEL*	carboxyl ester lipase	<1%
MODY 9	*PAX4*	paired box 4	<1%
MODY 10	*INS*	Insulin	<1%
MODY 11	*BLK*	B lymphocyte kinase	<1%
MODY 12	*ABCC8*	ATP binding cassette subfamily C member 8	<1%
MODY 13	*KCNJ11*	potassium voltage-gated channel subfamily J member 11	<1%
MODY 14	*APPL1*	adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper 1	<1%

Each gene is responsible for some specific clinical features. Identifying a pathogenic variant often allows predicting future health complications as the higher risk of a cardiovascular event, and can guide the clinician for the best therapeutic approach [15].

Although it is apparent that mutations in *GCK* and *HNF1A* are by far the most common pathogenic variants responsible for MODY, their frequency is slightly different according to the geographical location [17]. According to studies undertaken in several countries, pathogenic variants in *GCK* (MODY 2) are the principal cause of MODY in Spain, France, Italy [18], Germany, Austria [19], Japan [20], the Czech Republic [21], Poland, Italy, and Greece. However, in Denmark, the UK, the Netherlands [21], Norway [22], China [23], and Korea, the predominant is *HNF1A* (MODY 3). The explanation for this variation may be a genetic inheritance factor, but each country's screening strategies and the selection criteria for patients for genetic testing could be also the cause [22]. Therefore, countries routinely performing blood glucose tests have higher diagnostic rates for MODY 2. On the other hand, countries that seldom perform routine blood glucose tests have higher diagnostic rates for MODY 3 [11] [17] [18].

In Portugal despite data showing that the estimated prevalence of diabetes is higher than the global and European estimates [24], no statistics about monogenic diabetes are available since most MODY patients are often misdiagnosed with T1DM or T2DM [25]. Also, the genetic diagnoses that have been performed in Portugal are still underpowered to provide accurate

population estimates [22]. A recent study carried out in forty-six Caucasian Portuguese families showed that the most frequent genetic variations were found in the *GCK* gene, followed by defects in *HNF1A*. From the 4 most common MODY causes, *HNF4A* is rarer than the previous ones as expected according to their known contribution to MODY [26].

Until now, a great variety of pathogenic variants associated with MODY phenotypes have been identified such as missense, nonsense, splicing, promoter region variants, frameshifts, insertions and duplications, small, partial, and whole gene deletions [17] [27]. While in *GCK*, *HNF1A* and *HNF4A* is rather common to identify missense mutations [28] [29], in *HNF1B*, approximately 50% of cases are a consequence of whole gene deletions [30]. New sequencing approaches supported by the increasing advance in the genetics field seem crucial to the adequate MODY diagnosis. This is especially important not only to identify rare forms of monogenic diabetes, but also to provide a better understanding of the pathological process of the disease. Furthermore, a complete genetic diagnosis can support a correct diagnosis of an inconclusive patient with a clinical diagnosis of MODY [22].

GCK

The glucokinase gene (*GCK*) is located on the short arm of chromosome 7 (7p13) with around 46 kb divided into 12 exons encoding the 465-amino-acid protein, which is one member of the hexokinase family of enzymes [31]. Among several transcripts derived from alternative splicing, most isoforms are expressed in hepatocytes and pancreatic islet β-cells [32]. The pancreatic β-cell isoform of the enzyme is composed of 10 exons [31]. GCK is a monomeric enzyme composed of two domains: the large and the small domain. These two domains are separated by a deep cleft where the glucose binding site is located. Thus, amino acids 1 to 64 and 206 to 439 belong to the large domain, amino acids from 72 to 201 and from 445 to 465 belong to the small domain, and amino acids from 65 to 71, from 202 to 205, and from 440 to 444 belong to the three loops connecting the domains. The deep cleft is composed of residues Glu256 and Glu290 of the large domain, residues Thr168 and Lys169 of the small domain, and residues Asn204 and Asp205 of the connecting region I. Also, the protein conformation switch between three possible forms, super-open, open, and closed according to glucose concentration [28] [33] [34]. Figure 1 shows the *GCK* gene structure associated with the two principal functional domains.

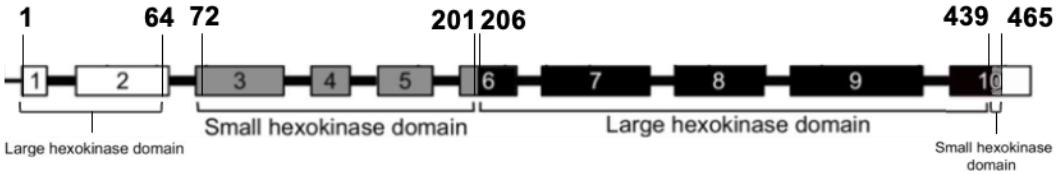

Figure 1 *GCK* gene structure. Numbered boxes correspond to exons. Numbers in bold are amino acid residue positions. The white (1 to 64) and the black section (206 to 439) represent the large domain, grey is the small domain (91 to 279 and 445 to 465). Adapted from *[35]*.

This enzyme plays an important role in the first step of glucose metabolism, catalysing the transfer of a phosphate from ATP to glucose, and generating glucose-6-phosphate (G6P). Thus, the concentration of glucose in the blood is directly proportional to the activity of GCK [11]. The isoforms that have been found in the liver are involved with the regulation of glucose homeostasis maintaining glucose synthesis, breakdown, transport, and storage [31] [36]. In its

turn, the glucokinase isoform identified in pancreatic β-cells is considered a "glucose sensor" stimulating insulin release according to changing the glucose phosphorylation rate proportional to physiological glucose concentrations [11] [31] [32]. Genetic defects along the 10 exons encoding this last referred isoform of the enzyme are responsible for MODY2 phenotype [31]. See supplementary material for the full *GCK* sequencing [36].

In 1992, the first cases of GCK-MODY were diagnosed in French and UK families [31]. Nowadays, over 800 variants are reported distributed throughout most of the coding regions and exon-intron boundaries of the *GCK* gene. Pathogenic *GCK* variants are present in 1 in 1000 individuals, although most cases of GCK-MODY are undiagnosed or misdiagnosed as T1DM or T2DM [37]. Until now, several genetic alterations have already been identified that directly influence clinical features and the respective treatment approaches. Heterozygous gain-of-function trigger to persistent hyperinsulinemia hypoglycaemia mutations through switch GCK to active open conformation leading a reduction of glucose levels [28] [39]. Otherwise, homozygous loss-of-function and compound heterozygous have more severe phenotypes presenting at birth permanent neonatal diabetes mellitus [31]. In these patients, the enzyme is completely deficient, requiring constant insulin therapy since the neonatal period [11].

Lastly, the most common alterations are heterozygous loss-of-function mutations, responsible for GCK-MODY phenotypes. Heterozygous loss-of-function mutations in GCK show lower phosphorylation levels, leading to decreased formation of G6P and, consequently, blood glucose accumulation. The higher glucose concentration leads to an insulin release with elevated glucose levels compared with non-diabetic subjects [11]. Thus, these patients show a raised threshold for the initiation of glucose-stimulated insulin secretion [9].

Furthermore, another factor to consider is the levels of glycated haemoglobin (HbA1c), which is a measure of the amount of glucose attached to circulant haemoglobin. The screening of HbA1c is widely used to manage the treatment and the risk of other health complications associated with diabetes. Elevated blood glucose concentrations facilitate the process of glycation of haemoglobin, increasing the levels of HbA1c. The reference value is between 4,0 and 5,6% for non-diabetic, although it is accepted until 8% for controlled diabetic patients. [39]. Usually, GCK-MODY patients have mild hyperglycaemia allowing them to maintain HbA1c levels below 8% what means they are within the range of stable glycaemic control. They often are diagnosed during routine screening for unrelated illness or during pregnancy since the absence of symptoms is very common. Also, there are no evidence to predict a higher risk of microvascular and macrovascular complications associated with diabetes [9] [11] [16].

Studies about pharmacological treatments used in patients with glucokinase pathogenic variants indicate that therapeutics with oral hypoglycaemic agents (OHA) or insulin may be inefficient. When these patients are on pharmacological treatment, their HbA1c levels do not decrease, which allows concluding no treatment is needed for most patients [42]. This unexpected result is due to a good response of the regulation of glucose levels, even though raised threshold. Thus, GCK-MODY patients, when are submitted to an oral glucose loading, rapidly falls towards the fasting level [42].

However, the discontinuation of pharmacologic treatments still remains unclear and should be individually evaluated. Some patients with unusual clinical features may still be required to prescribe OHA to better control hyperglycaemia [42]. On the other hand, strong evidence defends that exogenous insulin has a suppressor effect in endogenous insulin secretion.

Therefore, administering a small dose of exogenous insulin leads to a compensatory reduction in endogenous insulin secretion without alteration in glycaemic and HbA1c levels, making this therapeutic approach extremely inadvisable [15] [41].

As already mentioned, the identification of *GCK* deficient patients can be complex due to asymptomatic conditions. Moreover, the autosomal dominant pattern of inheritance may not be evident because of poor information about co-segregation data and lack of diagnoses of relatives [11]. During pregnancy, the identification of MODY caused by a *GCK* mutation in women has special relevance. Despite most patients not demanding pharmacologic treatment, mothers may take insulin therapy during pregnancy to prevent possible complications for the baby. This decision should be made whenever possible based on the known foetal genotype. From the genetic data, it is possible to predict how an insulin stimulus will affect foetal development [11] [15]. In the first scenario, the foetus does not have a *GCK* pathogenic variant that promotes an increase of insulin secretion leading to an excess growth as a response to maternal hyperglycaemia. Therefore, the mother should resort to insulin due to an imminent risk of baby macrosomia. In contrast, when the same variant in the foetus is detected, his development seems normal even without his mother using insulin. If the foetus inherits a *GCK* pathogenic variant from the father or has a *de novo GCK* pathogenic variant, their insulin production decreases, resulting in lower birth weight. Although there is a clear association between the foetus genotype and the pregnant therapeutic approach guideline, more studies are needed to produce stronger consensual evidence [11] [15] [16].

Clinical features of GCK-MODY

Most cases with pathogenic variants in *GCK* are non-obese young adults, teenagers, and children who often present an asymptomatic persistent mild hyperglycaemia. Furthermore, the correct diagnosis is essential for these patients leading them to receive appropriate treatment and management. The following features allow suspicion of a GCK-MODY phenotype [11] [41] [43]:

1. Persistent fasting hyperglycaemia between 99.0 – 144.0 mg/dL;
2. An oral glucose tolerance test glucose (OGTT) – 120 min glucose minus 0 min glucose – greater than 54.0 mg/dL;
3. Negative pancreatic autoantibodies;
4. A near normal HbA1c levels under 7.5%;
5. Persistent fasting C-peptide production (stimulated serum C-peptide upper 0.6 ng/mL);
6. One parent having mild fasting hyperglycaemia.

Usually, abnormal glycaemia in parents is not detected. Even though the absence of family history if the other features are verified, the patient should be indicated to the genetic testing to confirm a possible mutation in *GCK* [43].

HNF1A

HNF1A (hepatocyte nuclear factor 1-alpha) makes part of the hepatocyte nuclear factors genes, a group of transcription factors associated with long-term diabetic complications [11] [44]. It is localized on chromosome 12 (12q24.31) containing 10 exons. There were three alternative isomers (*HNF1A* (A); *HNF1A* (B); *HNF1A* (C)) reported. All isoforms are present in several tissues such as the kidney, liver, isolated islets, and pancreas, although they have different expression profiles concerning the tissue and the development state [45]. The isoform A encodes the longest proteins with 631 amino acid residues. Also, it shows higher expression

levels in the liver, kidney, and foetal pancreas, which suggests it has a special role in pancreatic development [45]. The isoform B contains 6 exons encoding 572 amino acid residues protein, while the isoform C has 7 exons expressing a protein with 524 amino acid residues. The predominant presence of isoform B in the mature pancreas supports its linkage with the continued maintenance of β-cell function [44]. Nowadays, there are notable differences in the relative spatial expression patterns and the possible specific influence of each isoform in each tissue, reflecting the need to activate different subsets of downstream effectors in these tissues. Studies evidence an apparent developmental switch from *HNF1A* (A) in the foetal pancreas to *HNF1A* (B) in the adult may contribute to prove the temporal or developmental variation in expression of *HNF1A* isoforms [45] [46].

The *HNF1A* protein has three functional domains: a dimerization domain composed of amino acids from 1 to 32, a DNA-binding domain with amino acids from 91 to 279, divided into two homeodomains, and a transactivation domain containing amino acids from 281 to 631 [47]. In figure 2 shows the *HNF1A* gene structure associated with each functional domain. *HNF1A* is located in the nucleus, particularly in the liver and pancreas cells, where it is involved in the transcriptional regulation of several genes. Most of them are associated with regular hepatic function binding at least 222 target genes in hepatocytes affecting carbohydrate synthesis and storage, lipid metabolism, detoxification, and synthesis of serum proteins. Also, it plays a crucial role in the central rate-limiting steps in gluconeogenesis and associated pathways [47]. In the pancreas, despite having an essential role during embryonic development of the pancreas, *HNF1A* is associated with the development, proliferation, and cell death of mature, regulating the transcription of many genes, including insulin (INS) and the glucose transporter molecule (GLUT2) [11] [18] [46] [48]. Defects in *HNF1A* lead to a reduction of β-cell proliferation and increased apoptosis, as well decreased renal glucose reabsorption causing glycosuria that is often one of the primary symptoms of patients with *HNF1A* pathogenic variants [11] [18].

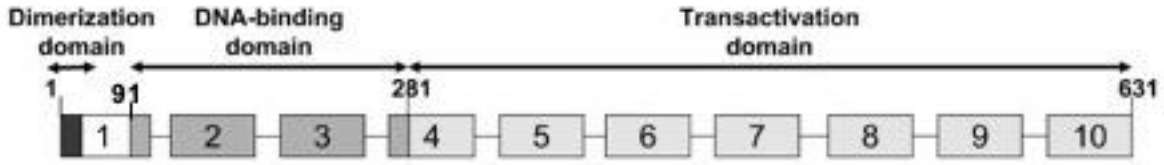

Figure 2 *HNF1A* gene structure. Numbered boxes correspond to exons. Numbers in bold are amino acid residue positions. The black section represents the dimerization domain (1 to 32), dark grey is the DNA-binding domain (91 to 279), and light grey is the transactivation domain (281 to 631). Adapted from *[29]*.

Heterozygous pathogenic variants in the *HNF1A* gene cause early-adulthood onset due to a progressive insulin deficiency. Until now, over 400 pathogenic variants were reported. Despite having been found in all exons, the more significant alterations are in exon 2 and 4. The most-reported pathogenic variants are missense mutations (55%), followed by frameshift (22%), splice site (9%), and promoter region mutations (2%), and deletions (1.2%) [42]. These mutations have a higher penetrance, with 63% of carriers developing diabetes by 25 years of age, 79% by 35 years, and 96% by 55 years, leading to HNF1A-MODY phenotypes [11].

Moreover, it seems to exist a correlation between the mutation region and the clinical phenotype. According to the isoforms of the gene, it is possible to divide into three mutation region groups: alterations between 1 to 6 exons, in exon 7, and between 8 to 10 exons [45]. Some evidence shows affect individuals who present pathogenic variants in the first half of the gene present symptoms around 8 years earlier than individuals affected 7-10 exon [18].

Actually, pathogenic variants in the 1-6 exons affect the three isoforms increasing the pathogenicity of a variant found between these exons [18].

Most cases of HNF1A-MODY manifest in adolescence or early adulthood before the age of 25 years [11] [47]. At birth, these individuals tend to be slim, having a normal sensibility to insulin. This type of MODY is characterized by a progressive β-cells dysfunction that leads to compromise of bioavailability of insulin [25]. Children are used to manifesting mild hyperglycaemia that becomes severe during their lifetime. In the early phase of diabetes, *HNF1A* carries, with good metabolic control, do not need exogenous insulin since the risk of ketoacidosis is small [18]. Despite having cases where the disfunction detection is premature, normally, it is later than 8 years of age [11] [42].

Studies during pregnancy corroborated strong evidence of the progressive dysfunction feature of HNF1A pathogenic variants. heterozygotic pathogenic variants do not manifested in the foetus. Insulin secretion in utero was not affected without influence the baby's birth weight. During development, the insulin levels decrease, triggering gradually aggravated hyperglycaemia states and complications associated with diabetes such as microvascular and macrovascular complications [18] [25] [42].

Oppositely to GCK-MODY, patients affected with HNF1A-MODY have an elevated risk of cardiovascular events due to poor glycaemic control. The risk associated with micro- or macrovascular complications of these patients is around the same of patients with Type 1 and Type 2 diabetes. When exposed to an oral glucose tolerance test, their show a great glucose rise (>90mg/dL). However, due to maintaining insulin levels to achieve euglycaemia in a fasting state, these patients often present a normal fasting plasma glucose concentration [11] [25].

As already mentioned, the *HNF1A* gene is expressed in different tissues, which leads to extra-pancreatic clinical features. In the kidney, the presence of deficient *HNF1A* reduces the expression of sodium-glucose cotransporter 2 (SGLT-2), resulting in the onset of glycosuria, a decrease of glucose reabsorption in the proximal tubule and consequently in a lower renal threshold for glucose. Glycosuria is one of the first features detected, often manifesting before the onset of hyperglycaemia. Moreover, through serum lipoprotein analysis of HNF1A-MODY patients, high-density lipoprotein (HDL) cholesterol levels are higher than T1DM and T2DM. Although this evidence could reduce the risk of cardiovascular events, it is not verified. Indeed, the risk of cardiovascular complications in these patients is higher than T1DM but lower than T2DM [11] [25]. Lastly, despite being rare, somatic *HNF1A* biallelic loss-of-function may trigger liver adenomatosis due to upregulated expression of proliferation and cell cycle regulatory genes secondary in hepatocytes [49].

Nowadays, it is known that the first-line therapy of patients with *HNF1A* pathogenic variants should be a low dose of oral hypoglycaemic agent sulphonylurea. These drugs bind to sulfonylurea type 1 receptor (SUR1), leading to the closure of ATP-dependent potassium channels and, consequently, raising the membrane potential of pancreatic β-cells. The variation of potential stimulates the opening of voltage-dependent calcium channels and an influx of calcium ions, promoting the fusion of insulin stored vesicles with the cell membrane increasing insulin secretion. This knowledge allows understanding the principles behind the sensitivity of *HNF1A* patients to sulphonylurea. However, this therapeutic approach should be appropriately evaluated due to the significant risk of hypoglycaemia when overdose exposure [50]. Besides, some studies show transference from insulin to sulphonylurea had a positive impact on HbA1c,

resulting in a decrease in the risk of diabetes-related complications [51]. Many patients had successfully transferred to sulphonylurea tablets without ketoacidosis and with good glycaemic control. This innovative therapeutical change allows a considerable patients' lifestyle improvement [11].

During pregnancy, the woman must have tight management of hyperglycaemia to maintain glycaemic control to reduce the risk of neonatal diabetes-related complications. There is no significant difference in congenital disabilities between women who took an oral hypoglycaemic and women using insulin to treat diabetes [15]. In some cases, other OHAs act on the same receptor as sulfonylureas could be efficient in treating HNF1A-MODY with a lower probability of hypoglycaemia. Furthermore, studies about the long-time effectiveness of sulfonylureas allow concluding a deterioration in glycaemic control over time what may require additional insulin therapy. Thus, the chosen method of any therapeutic approach should be evaluated case by case and considering the cost-effectiveness [15].

Clinical features of HNF1A-MODY

Despite having a great heterogeneity, HNF1A-MODY used to onset before the age of 25 years. The patients tend to be slim and normal glycemia during the neonatal period triggering the diabetes onset later due to progressive insulin release dysfunction. The following features allow the clinical suspicions of a defect in the *HNF1A* gene and request genetic testing [11].

1. An oral glucose tolerance test glucose (OGTT) – 120 min glucose minus 0 min glucose – greater than 90.0 mg/dL;
2. Negative pancreatic autoantibodies;
3. Persistent fasting C-peptide production (stimulated serum C-peptide upper 0.6 ng/mL);
4. HDL cholesterol upper 23.4 mg/dL;
5. Glycosuria at blood glucose <180.0 mg/dL

HNF1A-MODY patients still are misdiagnosed by T1DM or T2DM even though the distinctive known clinical features sometimes there are overlapping. Due to a strong family history of diabetes, when an individual is diagnosed with this type of MODY, genetic testing should be suggested to relatives with diabetes. Since pathogenic variants in *HNF1A* are often discovered as the cause of diabetes [43].

HNF4A

HNF4A (hepatocyte nuclear factor 4 alpha) belongs to the same transcription factors group of *HNF1A*. According to NCBI, the *HNF4A* gene is located on chromosome 20 (20q13.12), counting 13 exons considering the four variations of exon 1 (1A, 1B, 1C, and 1D) and two promoters (P1 and P2) [52] [54]. *HNF4A* is present in several tissues such as the liver, kidney, intestine, stomach, and pancreas. The P1 promoter controls the expression in most issues with predominant influence in the liver also drives expression in the foetal pancreas. Otherwise, the P2 promotor is 46 kb upstream of the P1 promoter and is responsible for the expression of *HNF4A* in the mature pancreas [52] [54]. There are well known 9 different isoforms of *HNF4A*, the 1 to 6 isoforms are associated with P1 promotor and hepatic function while 7 to 9 isoforms are expressed in the pancreas via P2 promotor. Also, the transcripts associate with the P2 promotor only contains exon 1D, which not happens with the P1 promotor transcripts as be represented in the figure 3.

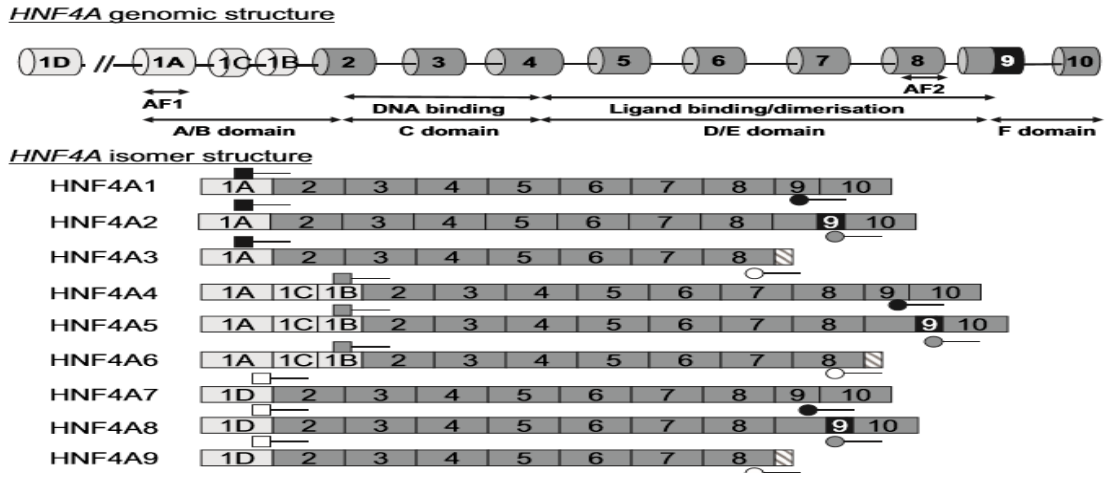

Figure 3: Isoforms of *HNF4A* gene. A – Whole gene structure with the four variations of exon 1 (1A-1D). B – Nine isoforms of the *HNF4A* gene. *HNF4A* 1 to *HNF4A* 6 isoforms associated with P1 promotor. *HNF4A* 7 to *HNF4A* 9 isoforms associated with P2 promotor. Alternate exons are coded in gray. The 10 –amino acid insertion present in exon 9 in isoforms *HNF4A* 2, *HNF4A* 5, and *HNF4A* 8 is marked in black. Intronic sequences included in isoforms *HNF4A* 3, *HNF4A* 6, and *HNF4A* 9 are indicated by hatched boxes. AF-1 and AF-2 refer to activation function motifs. The domain structure of the HNF4A protein and the positions of the isoform-specific probes used are given; probe set 1 is marked by black, gray, and white squares, and probe set 2 is marked by black, gray, and white circles. Adapted from *[52]*.

The *HNF4A* gene encodes a 465-amino acid protein with five function domains (A/B to F), which binds DNA as a homodimer [52]. This protein is expressed in several tissues having an important role in early development and cellular differentiation. Thus, the absence of *HNF4A* may compromise tissue formation. It is responsible for regulating several genes involved in essential processes such as gluconeogenesis, bile acid synthesis, and lipid metabolism pathway. Moreover, *HNF4A* play an important role in the control of the development and differentiation of mature β-cells. *HNF4A* takes part in the same complex transcription factor network of *HNF1A* acting on the INS promoter, directly affecting insulin secretion [52]. Thus, *HNF4A* is upregulated by some proteins, including the *HNF1A*, *PDX1*, and *HNF1B* gene products, and downregulated by itself. It is important to highlight *HNF4A* and *HNF1A* relation since their expression seems to be regulated mutually. Indeed, it is known variants in *HNF4A* generate similar phenotypes described in MODY 3 patients due to the autoregulatory relationship between the genes. However, some studies indicate a differential relation according to the state of development. A few features, such as hyperinsulinemia or macrosomia, are detected in *HNF4A* affected new-borns but are not associated with *HNF1A*. This type of evidence allows suspecting to the network between these two genes is different in adult and foetal β-cells [52] [54].

Variants in the *HNF4A* gene are located over the exons and the P2 promoter. These are responsible for HNF4A-MODY phenotypes representing around 3-10% of MODY cause [11] [18]. Due to sharing the same transcription pathway, these phenotypes are characterized by a progressive β-cell dysfunction like happen on deficient *HNF1A*. Concerning the penetrance of *HNF4A* pathogenic variants is lower and more variable than *HNF1A*. Despite most cases occurring by 25 years, there are patients without signals of onset diabetes even after 40 years [11]. Until now, over 100 pathogenic variants have been identified in *HNF4A*, more than 50% are missense mutations, followed by frameshift, nonsense, splice site mutations, promoter region mutations, insertions, duplications, and partial or whole gene deletions. The most frequent pathogenic variants are detected at exons 7 and 8 [27]. Also, the location of the variant

seems to influence the age of diagnosis according to the affected isoforms. Previous studies showed alterations in exons 9 and 10 are associated with a later onset of diabetes when compared with alterations in other exons [52].

As has been already mentioned, the phenotypical features of *HNF4A* and *HNF1A* are very similar, as such, MODY patients with *HNF4A* affected also tend to the development of microvascular complications. Therefore, in the presence of strong clinical feature evidence of MODY phenotype and *HNF1A* is negative, the genetic testing to HNF4A-MODY is highly recommended [18] [42]. Even though HNF4A-MODY patients are associated with divergent features from *HNF1A*, sush as, neonatal complications as foetal macrosomia and transient neonatal hyperinsulinemic hypoglycaemia, the absence of glycosuria and low levels of apolipoproteins (apoAII, apoCIII, and apoB) [18] [42].

Regarding pathological process and mechanisms of action of drugs are very similar to *HNF1A* deficient. Therefore, sulphonylureas should be the best treatment approach for *HNF4A* patients since a low-dose seem to be very efficient in glycaemic control [15] [50].

The pregnancy management is rather relevant when one of the parents has MODY due to a *HNF4A* pathogenic variant since there is risk of an excess weight of the newborn [27]. Heterozygous pathogenic variants lead to an increase of insulin secretion, triggering hyperinsulinaemic hypoglycaemia, and, consequently, macrosomia (around 800g raise in body weight). Also, a gradual decrease of endogenous insulin production is obverse from childhood until the onset of diabetes [11] [18] [41]. Whenever possible, the assessment of the genetic information of the foetus is recommended to better management during pregnancy to prevent neonatal complications [27].

Clinical features of HNF4A-MODY

Since *HNF4A* is present in several tissues, it makes alterations in this gene to produce a few extra-pancreatic features. Lower concentrations of total HDL cholesterol, apolipoprotein A1, apolipoprotein A2, and triglycerides is common. The following clinical features are often observed in this type of MODY patients being evidence for the genetic testing prescription [11].

1. An oral glucose tolerance test glucose (OGTT) – 120 min glucose minus 0 min glucose – greater than 90.0 mg/dL;
2. Negative pancreatic autoantibodies;
3. Persistent fasting C-peptide production (stimulated serum C-peptide upper 0.6 ng/mL);
4. Low HDL cholesterol and low triglyceride.
5. Macrosomia and/or neonatal hyperinsulinaemic hypoglycaemic.

Additionally, the genetic testing of *HNF4A* should be considered in macrosomia birth or familiar macrosomia mark (upper than 4.4 kg) or if diazoxide-responsive neonatal hyperinsulinism has been diagnosed [42] [43].

HNF1B

HNF1B is a transcription factor of the homeodomain-containing superfamily of transcription factors, like *HNF1A* and *HNF4A*. This transcription factor is present in the nucleus can act as a homodimer or a heterodimer playing a role in regulating gene expression. The *HNF1B* gene is in chromosome 17 (17q12). It is composed of 11 exons leading to three different isoforms. This protein has three functional domains: the dimerization domain composed of amino acids 1 to

32, the DNA binding domain containing amino acids 90 to 311, and the transactivation domain with amino acids 312 to 557 [55] [56]. Figure 4 shows the *HNF1B* gene structure associated with each functional domain.

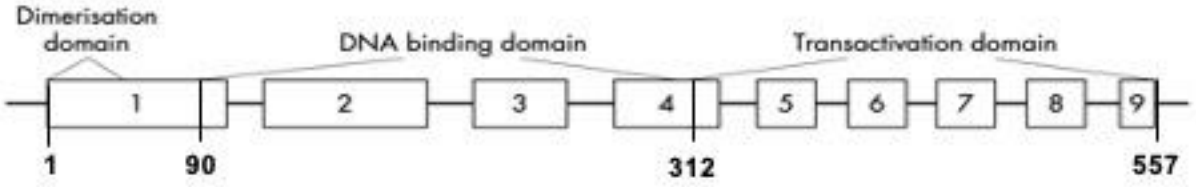

Figure 4 *HNF1B* gene structure. Numbered boxes correspond to exons. Numbers in bold are amino acid residue positions. The domain design is similar to *HNF1A*, the dimerization domain (1 to 32), the DNA binding domain containing (90 to 311), and the transactivation domain (312 to 557). Adapted from [56].

Curiously, *HNF1B* is expressed in the early stage of embryonic development in different tissues as the thymus, lung, kidney, liver, pancreas, bile ducts, stomach, intestines, and genital tract. Due to their multi-systemic action, *HNF1B* has an essential role in normal development and embryonic survival. Indeed, between a considerable number of HNF1B-associated complications it is essential to highlight the relation of this transcription factor with the nephron development leading to the early onset of renal disease. Additionally, the *HNF1B* plays a role in embryonic pancreatic development seems to explain the association with MODY phenotypes [55].

Pathogenic variants in *HNF1B* represent around 1–5% of all MODY cases [15]. The penetrance is highly variable, resulting in a large range of age at diagnosis (0 to 61 years). In contrast with *HNF1A* or *HNF4A*, strong evidence shows that the pathophysiology of HNF1-MODY is not only reflection of β-cells dysfunction but the combination of it with insulin resistance [11] [55]. Until now, more than 65 heterozygous variants have been reported, including missense, nonsense, frameshift, and splicing variants. The first four exons of the gene have a higher mutation frequency since exon 2, exon 4, and the intron 2 splice site are the most common affected sites. Interestingly, large deletions in HNF1B-MODY patients are often detected. Around half of the affected individuals show large rearrangements, exon deletion, or even whole-gene deletion [18] [54]. Although *HNF1B* has an autosomal dominant inheritance pattern characteristic of MODY, usually affected patients might have no family history of renal disease or diabetes. Consequently, spontaneous de *novo* mutations are common [11] [18] [55].

As already mentioned, the *HNF1B* activity manifests in early embryonic development in several tissues. Thus, the presence of *HNF1B* deficiency triggers irreparable damage in the organs' structures. Chronic kidney disorders are the principal feature of HNF1B-MODY; indeed, it is more frequent than diabetes. The patients who are often indicated to genetic testing have been already diagnosed with renal problems. Moreover, around 50% of affected individuals require renal replacement before 45 years due to end-stage renal failure [11] [15] [55]. Even though the predominant kidney disorder diagnosed in HNF1B-MODY patients is renal cysts, other structural abnormalities have been reported as renal hypoplasia, horseshoe kidney, and duplex kidney. Also, the malformations of the kidney promoted by *HNF1B* may result in hypomagnesaemia and hyperuricaemia [55].

Abnormalities in *HNF1B* have consequences in the development and differentiation of the pancreas, reducing endocrine and exocrine function. Truly, the early onset of diabetes is the extra-renal features most identified in children with *HNF1B*-associated disorder. Some data

corroborate the hypothesis of individuals with *HNF1B* variants present congenital underdevelopment of the pancreas. Additionally, pancreatic hypoplasia (a diminished or even the absence of pancreatic structures) is responsible for β-cells dysfunction and consequently a deficient insulin secretion. On the other hand, the low insulin levels in utero lead to a birth weight decrease of around 800g [11] [54]. Moreover, some evidence shows pancreatic exocrine hypersecretion as a compensatory mechanism for the reduced size of the pancreas [55].

Many extra-pancreatic features have been identified, highlighting genital malformations, especially in females, abnormal hepatic function with the asymptomatic rise of liver enzymes, and the higher risk of microvascular complications [15] [18]. Furthermore, recent studies about heterozygous contiguous deletion at chromosome 17q12 that includes *HNF1B* association show a linkage with neurologic features, namely, autism spectrum disorder (ASD) and cognitive impairment. Still, more studies are required to define this possible relationship [15] [55].

Although some similarities with the other hepatocyte nuclear factors, *HNF1B* patients should have a treatment approach different than the previous ones. These individuals typically present insulin resistance, which does not allow them to switch over from insulin therapy with efficient glycaemic control [50]. Considering the multi-systematic association of *HNF1B*, glucose adequate control is crucial to prevent the worsening of several health problems.

Clinical features of HNF1B-MODY

HNF1B-MODY is clinical heterogeneity, and the common absence of family history makes the clinical prediction an embarrassment leading to an under indication for genetic testing. Even though the following features are a strong clue of a possible genetic alteration in *HNF1B* [11] [55].

1. An oral glucose tolerance test glucose (OGTT) – 120 min glucose minus 0 min glucose – greater than 90.0 mg/dL;
2. Negative pancreatic autoantibodies;
3. Persistent fasting C-peptide production (stimulated serum C-peptide upper 0.6 ng/mL);
4. Renal impairment associated with creatinine increased, hyperuricaemia and hypomagnesaemia;
5. Higher levels of liver enzymes.

Moreover, genetic testing should be considered for all individuals presenting abnormalities *HNF1B*-associated tissue development, highlighting when they have been diagnosed with renal disease [55].

Rare types of MODY

Despite representing a lower frequency, some evidence supports the importance of detecting variants in less common MODY-associated genes [22]. Negative genetic testing for the four most common genes does not discard the possibility of a MODY phenotype. Rare forms of MODY genes show very similar phenotypical features with the most frequent MODY genes. These genes can also alter the normal pancreatic gene expression, leading to β-cells dysfunction or impairment of insulin release [11]. Additionally, in some cases, it seems to have a network linkage between them [11] [15] [18].

Emerging studies that can include a larger number of genes, as whole genome, or exome sequencing, allow an overall comprehension of MODY aetiology and can improve prevalence

studies and determine characteristics of MODY patients more accurately, improving the selection criteria for molecular genetics testing [22].

The importance of MODY diagnosis

The distinction between MODY and other types of diabetes is very relevant. Through molecular genetic testing, the clinician manages to select a better therapeutical approach based on aetiology, avoiding unnecessary and ineffective treatments. Moreover, often relatives carry the same mutation previously detected and so more patients at risk can be identified. Thus, the genetic diagnosis is beneficial for both patients and their relatives [9] [22].

Nowadays, some effort has been made to reduce the misdiagnosis, particularly with the design of next-generation sequencing (NGS) panel with a panel of MODY-associated genes. Despite decreasing costs and increasing capabilities, this procedure remains inaccessible for some countries, making Sanger sequencing the current protocol for this type of diagnosis. In the next few years, it is expected a progressive improvement of identification and diagnosis of MODY patients providing personalized medicine and more individualized treatment approaches. Also, the prediction and prevention of health complications according to the genetic information of each patient may provide a significant improvement in their life quality [22].

The main aim of this project is focused on the genetic identification and characterization of patients with a clinical diagnosis of MODY. DNA samples of the patients are studied through PCR amplification and Sanger sequencing of *GCK*, *HNF1A*, and *HNF1B* genes. Also, Multiplex Ligation-dependent Probe Amplification (MLPA) is performed to detect large rearrangements. Identifying the pathogenic variant responsible for the MODY phenotype allows adjusting a better treatment for each patient to prevent possible complications.

Material and Methods

1. Selection and sampling of patients

Nineteen subjects previously referred by *Associação Protectora dos Diabéticos de Portugal* (APDP) were selected. All the selected individuals had been diagnosed with diabetes and presented clinical features that met MODY selection criteria according to best practice guidelines defined by the European Molecular Genetics Quality Network [17]. They must have been diagnosed with early onset of diabetes (before 25 years old) with some evidence of the absence of pancreatic antibodies and no-signal of insulin resistance. Also, all of them presented strong family history of diabetes. It was also asked for the inclusion of relatives in the study, if possible.

To get more information about the patients, some data was collected through a questionnaire including the following parameters: sex, age of diagnosis, birth weight, BMI, OGTT, HbA1c, pancreatic antibodies, what type of treatment they were taking and diabetes-related clinical complications, such as renal disease, retinopathy, coronary disease, neuropathy, and nephropathy.

2. DNA extraction

The DNA extraction was made from the whole blood of subjects collected in ethylenediamine tetraacetic acid (EDTA) tubes using the salting-out method [57]. The DNA samples were stored at 4 ºC. To ensure reliable results, the DNA concentration was assessed through the average measured in the NanoDrop spectrophotometer. Attempts had been made to samples achieving concentrations between 100 ng/µl and 400 ng/µl. The quality of the DNA samples was verified by performing electrophoresis on 1% agarose gel (1.0 g agarose for 100mL 1X TBE Buffer diluted from UltraPure™ 10X TBE Buffer and 2 µL SYBR™ Safe DNA Gel Stain) for 30 minutes at 90 volts. The loading mix was composed of 5 µL loading buffer (bromophenol blue + glycerol) and 5 µL sample. The gels were evaluated under UV light with Safe Imager™.

3. PCR amplification of MODY genes

According to the clinical features, the probands were divided into two categories of study. One group was composed of subjects with renal complications were studied for *GCK*, *HNF1A*, and *HNF1B*. The remaining individuals were examined for *GCK* and *HNF1A* variants.

Thus, specific primers previously designed were used for each exon of target genes. For PCR, working solutions were made from primers stock solutions at 10 ρmol/µL and 20 ρmol/µl. For PCR reactions Bioline reagents (dNTP's, 10x NH4-based reaction buffer, 50 mM $MgCl_2$ solution, and BIOTAQ™ DNA Polymerase) or Applied Biosystems reagents (dNTP's, 10x Buffer I and AmpliTaq™ DNA Polymerase), kept stored at -20 ºC.

The exons amplification was performed as stated by optimal conditions to perform Polymerase chain reaction. However, some fragments required a few optimizations and adjustments as is presented in the table S1 of supplementary material. Starndard PCR mix and cyclig programs are presented in table S2 and S3 of of supplementary material.

To verify the success of reactions, the PCR products were submitted to electrophoresis in 2 % agarose gel. The gel was composed of 2.0 g agarose, 100mL 1X TBE Buffer (diluted from UltraPure™ 10X TBE Buffer) and 2 µL SYBR™ Safe DNA Gel Stain. Wells were loaded with a 5 µL loading buffer and 5 µL PCR product. The confirmation of amplification size was accessed by Thermo Scientific GeneRuler 100 bp DNA Ladder. The electrophoresis ran for 40

minutes at 90 volts. Afterwards, the agarose gels were evaluated under UV light with Safe Imager™.

4. Gene sequencing

PCR products were purified following the ExoSAP-IT™ PCR Product Clean-up protocol before carried out sequencing reactions. For Sanger sequencing reactions, the BigDye® Terminator v1.1 Cycle Sequencing kit was used with primers solutions at 2 ρmol/μL. Each reaction was added 1 μL of the purified PCR product. At table S4 of supplementary material is illustrated in detail the sequencing mix and the cycling program accomplished. After this first reaction, the fragments were submitted to capillary gel electrophoresis producing a sequencing chromatogram that was analysed according to the following description.

5. Sequence analysis and interpretation

For analysing the obtain chromatograms, it was required bioinformatics tools available at the lab, namely Staden Package software (Pregap4 and Gap4). Pregap4 was used to align the chromatograms for each fragment while using Gap4 to compare the fragments against each other and respective target gene reference sequences present in online databases. Reference Sequences used: *GCK* (NG_008847.2; NM_000162.5; NP_000153.1. NM_000545); *HNF1A* (NG_011731.2; NM_000545.8; NP_000536.6); *HNF1B* (NG_013019.2; NM_000458.4; NP_000449.1)

The nomenclature of all found variants was checked through Mutalyzer software. Following in silico analysis was performed with MutationTaster, PolyPhen-2, PROVEAN/SIFT, REVEL, NNSLIPE and MAXENTSCAN software to predict their pathogenicity. Also, each variant was searched in open online databases such as NCBI's dbSNP, PubMed, ClinVar, and Human Gene Mutation Database (HGMD). Lastly, variants were classified following American College of Medical Genetics and Genomics (ACMG) guidelines [58].

6. MLPA

To verify the presence of copy number variation (CNV) and methylation status of the target DNA on samples non-detected in Sanger sequencing resorted Multiplex Ligation-dependent Probe Amplification (MLPA) assay, according to MS-MLPA® protocol version MSP-v004 by MRC-Holland. MLPA was based on PCR principle, useful for the detection of genetic abnormalities such as aneuploidies, gene deletions or duplication, subtelomeric rearrangements and methylation status. MLPA probemixes had probes that target a specific genomic sequence. Each MLPA probe had a unique length, which is ligated in a target sequence and amplified by PCR. In the following step, PCR products were separated by length through capillary electrophoresis. The results were analysed and interpreted using, respectively, CoffalyzerNet software and open online databases.

Results

1. Subjects' characterization

In this study, twenty-nine individuals from nineteen different families were evaluated. Fifteen out of these were males (51.72%) and fourteen were females (48.28%). The study sample was composed of 68.97% caucasians and 20.69% of african descent. There was no data on the ethnic group in 10.34%. There were nineteen probands and ten relatives. The proband group was composed of 10 males (52.63%) and 9 females (47.37%). The ranged age was from 16 to 81 years old, with a mean of 52.37 ± 17.51 years and median of 56 years. Data about probands' age at diagnosis varied between 15 and 45 years with a mean of 29.28 ± 10.19 years and median of 30.5 years. There was not available information for one individual. As for relatives, they included five males (50.00%) and equally five females (50.00%). The age varied from 8 to 47 years old, with a mean of 28.30 ± 14.17 years. Despite most of the subjects not being diabetic, two individuals were diagnosed at 22 and 47 years.

2. Family history of diabetes and renal disease

All families presented a positive family history of diabetes. One proband and one relative non-related had gestational diabetes. On the other hand, only two out of nineteen (10.53%) had a clear family history of renal disease. The remaining seventeen families had no apparent family history of renal, or this information was not available.

3. Vascular complications

Diagnosis of vascular complications, commonly diabetes-related, namely, coronary disease, retinopathy, neuropathy, and nephropathy, was collected from subjects. Most of the probands had one or more than theses already mention pathologies, with only four out of nineteen (21.05%) had no vascular complication. One out of nineteen (5.26%) was diagnosed with coronary disease. Three out of nineteen (15.79%) presented retinopathy. One out of nineteen (5.26%) had nephropathy. One out of nineteen (5,26%) had been diagnosed with coronary disease and neuropathy. Also, one had both coronary disease and nephropathy. Two out of nineteen (10.53%) presented retinopathy and nephropathy. One out of nineteen (5.26%) was detected coronary disease, retinopathy, and neuropathy. Equally, only one subject presented coronary disease, neuropathy, and nephropathy. Two out of nineteen (10.53%) had retinopathy, neuropathy, and nephropathy at the same time. Also, two subjects (10.53%) had been diagnosed with all these vascular complications. Available data among relatives' group showed two individuals were affected with retinopathy and nephropathy, respectively. The remaining eight out ten (80%) were vascular complications free.

4. Birth weight and Body Mass Index (BMI)

According to obtained data from eight probands (42.11%), the gestation time was standard, around 40 weeks. The birth weight ranged from 3.100 kg to 4.900 kg, with a mean of 3.480 ± 0.620 kg. Only one individual (5.26%) had macrosomia weighing more than 4.000 kg. Seven out of nineteen (36.84%) were born with an average weight, between 2.500 kg to 3.999 kg. The remaining eleven individuals (57.89%) it was not allowed access to that data. Birth weight data was rare in relatives, only available for one subject who was born with 1.700 kg, lower weight than the normal range.

Whenever possible, BMI was recorded at the diagnosis and at enrolment for this study. Among probands, BMI at diagnosis was between 21.4 and 43.1 kg/m^2 with a mean of 27.51 ± 6.03 kg/m^2. Five out of nineteen (26.32%) had BMI values between the normal range, 18.5 to 24.9

kg/m^2. Nine (47.37%) presented BMI above 25 kg/m^2, and none presented low BMI. Data was not available for five individuals. Fortunately, it was possible to calculate BMI for all probands at enrolment. Thus, BMI at enrolment range from 19.4 to 37.3 kg/m^2, with a mean of 26.87 ± 5.25 kg/m^2. Nine of them (47.37%) had normal BMI, and the remaining ten (52.63%) had high BMI. Like of diagnosis, no one pointed out a BMI value below 18.5 kg/m^2. The available data indicated a BMI at enrolment of 22.4 kg/m^2 for one of the relatives.

5. Blood glucose measurement

Like BMI, fasting plasma glucose (FPG) and Hb1Ac levels were considered at two different measurements, at diagnosis and at enrolment. At diagnosis, five out of nineteen probands (26.32%) did not have available data. Considering the remaining fourteen individuals, FGP ranged from 150 to 500 mg/dL, with a mean of 314.64 ± 115.27 mg/dL and median of 300 mg/dL. All of them presented FGP over 126 mg/dL that characteristic of diabetes patients. Hb1Ac data were available for four out of nineteen (21.05%) ranging from 9.3 to 12% with the mean of 10.55 ± 1,17% and median of 10.5%. These values supported the diagnosis. At the second measurement, the overall range founded was a little lower from 72 to 309 mg/dL, with a mean of 178.63 ± 60.62 mg/dL and median of 176 mg/dL (table 2). Despite fourteen individuals (73.68%) continuing upper to 126 mg/dL, three (15.79%) presented FGP between 100 and 125 mg/dL. Moreover, two individuals (10.53%) were screened with under 100 mg/dL FPG levels (table 2). Hb1Ac levels at enrolment verified a reduction compared with the previous ones with values between 5.9 and 9.1% with a mean of 7.30 ± 0.94% and median of 7.2%. Five out of nineteen (26.32%) presented less than 6.5%, eight (42.11%) showed values between 6.5 and 7.5% which evidence well-control blood sugar levels for diabetics. Six probands (31.58%) had poorly controlled diabetes with HbA1c upper to 7.5% (table 3). It was not possible to assess oral glucose tolerance teste (OGTT) data of any probands.
One relative was evaluated with an impaired fasting glucose of 126 mg/dL even though Hb1Ac and OGTT levels were normal with values of 5.5% and 121 mg/dL, respectively. The remaining relatives had no available data.

Table 2 Blood glucose measurement from probands at diagnosis and at enrolment.

	FGP (mg/dL)		HbA1c (%)	
	At Diagnosis	**At Enrolment**	**At Diagnosis**	**At Enrolment**
Minimum value	150	72	9.3	5.9
Maximum value	500	309	12.0	9.1
Average	314.64	178.63	10.55	7.30
Standard deviation	115.27	60.62	1.17	0.94
Median	300	176	10.5	7.2

Table 3 Blood glucose levels at enrolment by categories. N represents the number of patients in each category. FGP between 100 and 125 mg/dL indicates higher risk of diabetes. FGP upper than 125 mg/dL indicates diabetes. Hb1Ac between 6,5 % and 7,5 % indicates well control diabetic patient. Hb1Ac upper than 7,5% indicates poor controlled diabetes.

Measurement	N	Relative Frequency (%)
FGP		
=<100 mg/dL	2	10,53
[100,125] mg/dL	3	15,79
>125 mg/dL	14	73,68
HbA1c		
<=6,5%	5	26,32
6,5% and 7,5%	8	42,11
>7,5%	6	31,58

6. C peptide and pancreatic antibodies

Subjects were screened for the presence of C peptide and pancreatic antibodies, glutamic acid decarboxylase antibodies (GADA), and islet cell antibodies (ICA). Reference value of C peptide range between 0,5 and 2 ng/mL. Among probands, C peptide values range from 0.37 to 6.99 ng/mL with a mean of 2.70 ± 2.08 ng/mL. Seven out of nineteen (36.84%) had C peptide between 0.5 and 2 ng/mL, one (5.26%) had lower than 0.5 ng/mL and seven individuals presented values higher than 2 ng/mL. Besides, four of them (21.05%) had no available data. The pancreatic antibodies analysis resulted in all negative except for one proband who was positive for GADA, which was suggested as a T1DM case. However, two subjects still were waiting for testing results, and another three did not provide available data. In the relatives' group, there was available data from one subject who presented a C peptide of 2.33 ng/mL and negative for pancreatic antibodies.

7. Treatment type

This study was considered three treatment types, diet, OHA, and insulin administration. Among the probands group, fifteen subjects (78.95%) were on a diet. Sixteen out of nineteen (84.21%) were on OHA. Also, data for the type of drug they were taking was available for twelve subjects. The most taken OHA drug is dulaglutide with 37.5%, followed by metformin with 18.75%, liraglutide, dapagliflozin, and gliclazide represented 12.5% each. There were fourteen (73.68%) on insulin therapy. However, seventeen probands had a combined therapeutical approach. Three subjects (15.79%) were on both diet and OHA, three were on both diet and insulin, two (10.53%) were on OHA and insulin, and nine (47.37%) were on a diet, OHA and insulin at the same time. Data about relative were available from two individuals even though only one were on treatment, diet and OHA.

8. Molecular Analysis

Sanger Sequencing analysis

It was detected twenty variants by Sanger sequencing. Nine were benign, two were likely pathogenic, two variants were pathogenic, and seven of them were classified as uncertain significance. In *GCK*, three variants were detected, one benign and two classified as uncertain significance. In *HNF1A*, thirteen variants were identified. Between them, five had uncertain significance. Lastly, no *HNF1B* variants were detected (figure 5). Additionally, no variants were detected via MLPA in any studied gene.

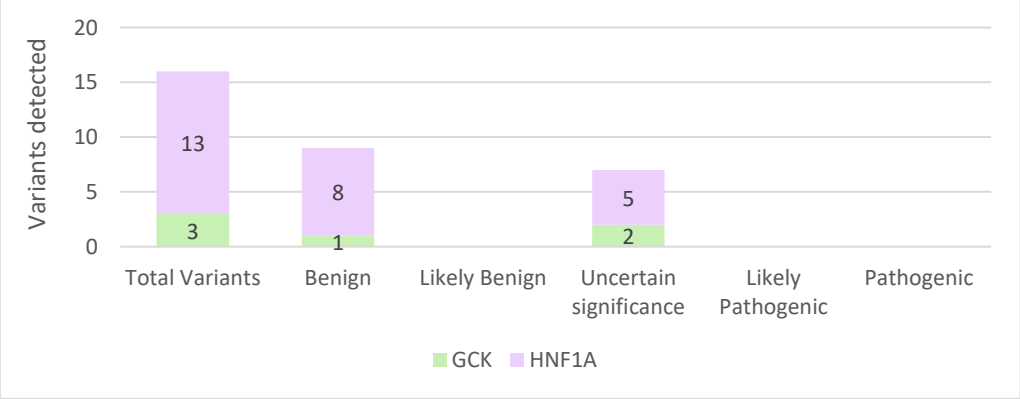

Figure 5 Detected variants and classification. Total of sixteen variants were found. Three in the *GCK* gene and thirteen in the *HNF1A* gene, as it is represented in the first column. Variants are distributed in the columns according their ACMG

classification. Among the three *GCK* variants one was benign and two as uncertain significance. In *HNF1A*, eight variants were benign and five were of uncertain significance.

GCK

A total of three variants were detected in the *GCK* gene. All variants were nucleotide substitutions. One of them, c.129C>T, was in the coding region in exon 2, while another two were in the upstream transcript region (figure 6). Curiously, the most common variant was c.-516G>A had been identified in 3 probands (figure 7).

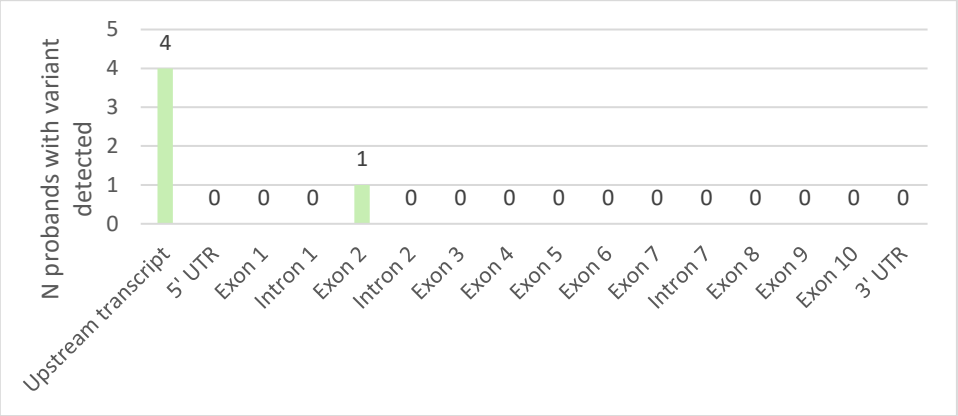

Figure 6 Number of probands with the different *GCK* variants by location. A total of 5 participants were found to have variants in the upstream region and in exon 2.

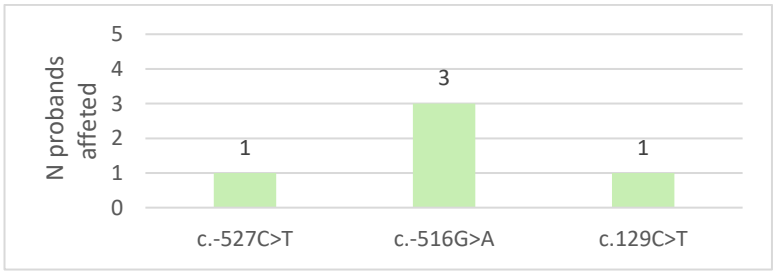

Figure 7 *GCK* variants. Number of probands affected with each found variant. Variants c.129C>T was synonymous variant while c.-527C>T and c.-516G>A were upstream transcript variants.

The variant, c.129C>T (figure 8), was located in exon 2. It was a single cytosine exchange to thymine on the third position of codon 43 (p.Arg43Arg). All *in silico* programs confirm no effect on protein structure, except MutationTaster that had a divergent prediction. This variant was present in ClinVar and dbSNP, although it had not already described in the literature. Also, there was no functional evidence. According to gnomAD, minor allele frequency (MAF) total of 0.002%, and the MAF in the East Asian population of 0.005% (table S5). Relatives samples were not available to carry out cosegregation assays. Variant c.129C>T was classified as uncertain significance by ACMG classification (table 4).

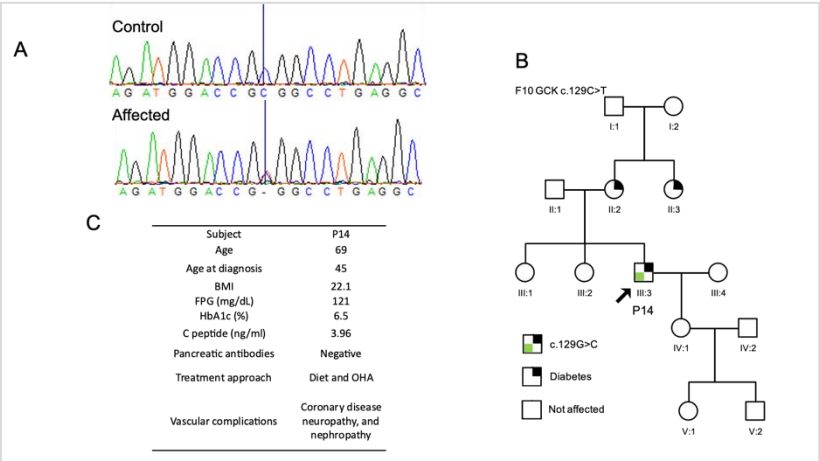

Figure 8 *GCK* c.129C>T chromatogram, pedigree and subject data. A: Representation of control and variant. B: Pedigree of family number 10. The arrow indicates the proband subject. C: Sum up of feature of subject P14. FPG measurement at enrolment.

The variant, c.-527C>T (figure 9), was a cytosine to thymine substitution located in the regulatory sequence of the *GCK* gene. As a non-coding alteration, it is essential to evaluate its effect on transcription and protein function. *In silico* evaluation evidence did not affect the RNA splicing. According to gnomAD, MAF total of 0.20%, and the MAF in the African or African American population of 0.69% (table S5). This variant was only present on dbSNP. Nevertheless, it was not described in the literature. Also, no data about cosegregation was available. The insufficient data only allow to classified c.-527C>T as uncertain significance by ACMG classification (table 4). Lastly, c.-516G>A variant was found data on online databases and literature which supported benign classification.

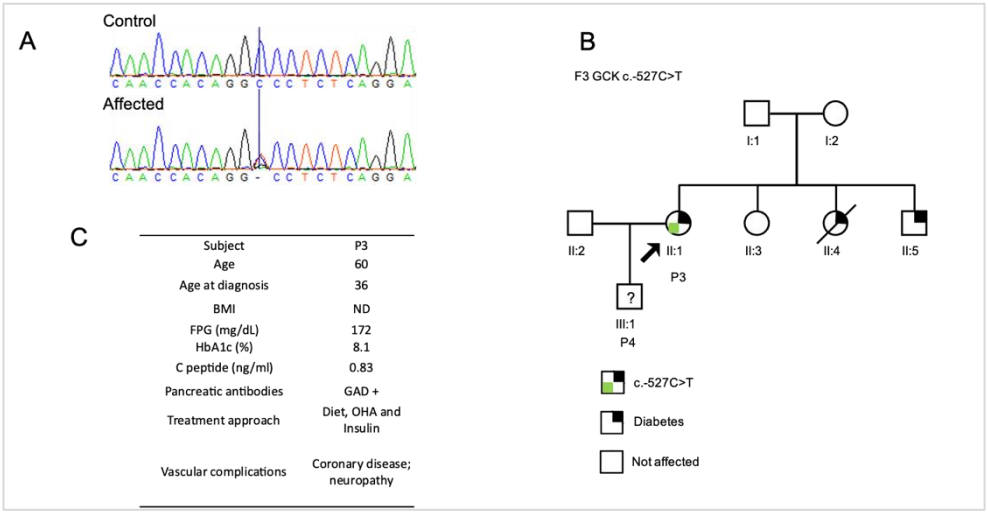

Figure 9 *GCK* c.-527CT chromatogram, pedigree and subject data. A: Representation of control and variant. B: Pedigree of family number 3. The arrow indicates the proband subject. Subject P4 showed threshold FPG levels. C: Sum up of feature of subject P3. FPG and HbA1c measurement at enrolment. ND meaning not diagnosed.

HNF1A

Most *HNF1A* variants were located in coding regions (77%), with only four out of thirteen in non-coding regions (23%). In the coding regions, there are found variants among exons 1, 4, 6, 7, and 8. On the other hand, variants in non-coding regions were located in intron 1, 2, and 7. Curiously, 37% of founded variants in *HNF1A* were located in exon 7 (figure 10).

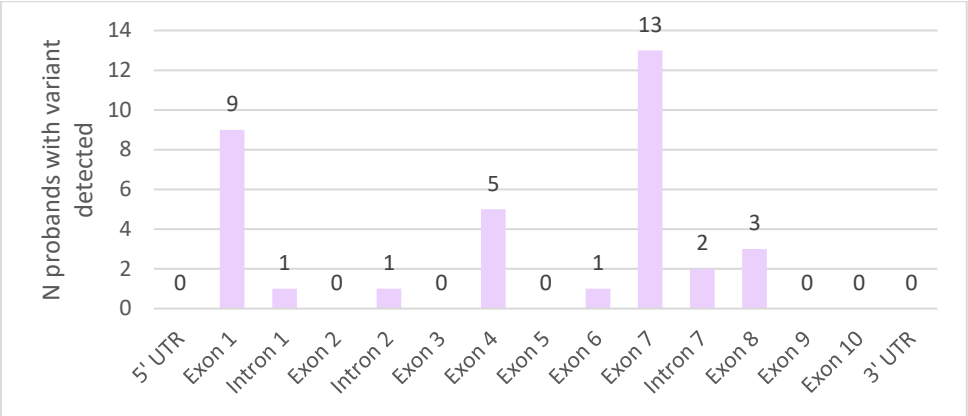

Figure 10 Number of probands with the different *HNF1A* variants by location. A total of 35 participants were found to have variants in 5 exons and 3 introns.

The overwhelming majority of founded variants were point substitutions alterations. Seven out of thirteen had not functional alteration, being synonymous variants. Three of them were missense, and the remaining three could impact splicing levels since there were located in intronic regions. The most common variant, c.1460G>A, is present in 7 probands, and it was located in exon 7 (figure 11).

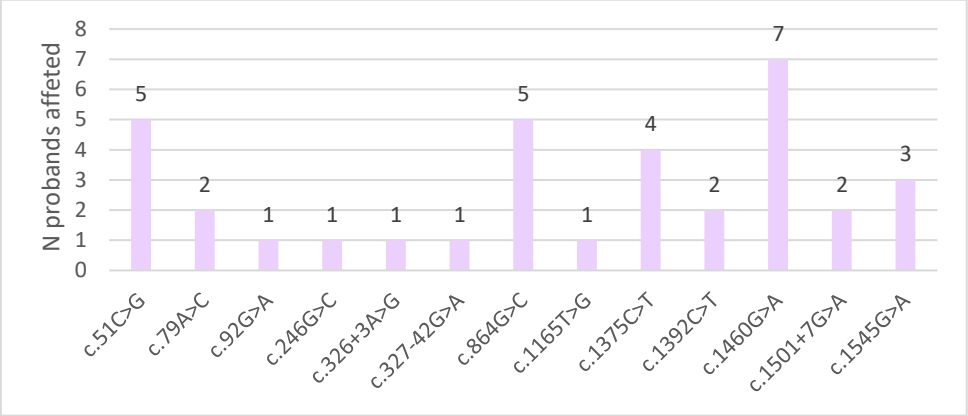

Figure 11 *HNF1A* variants. Number of probands affected with each found variant. Variants c.1165T>G, c.79A>C, and c.92G>A were missenses. Variants, c.1501+7G>A, c.326+3A>G, and c.327-42G>A could affect mRNA splicing. The remaining variants were synonymous.

Among *HNF1A* variants, five were classified as uncertain significance. The variant, c.92G>A (figure 12), was a missense variant located in exon 1 that altered the second nucleotide in codon 31 (GGT>GAT). This nucleotide substitution resulted in wild-type glycine substitution by aspartic acid, p.(Gly31Asp). This variant was described in the literature [58] [35] and it was presented in disease databases, namely HGMD, ClinVar and dbSNP. *In silico* analysis was inconclusive since PROVEAN, SIFT, and PolyPhen-2 predicted no impact but MutationTaster and REVEL predicted as disease causing. According to gnomAD, the MAF total was 0.08% and the MAF in Latino or Admixed American population of 0.16% (table S5). Data on relatives were not available to ascertain cosegregation. Also, there was no functional evidence for this variant on databases or literature. Even though the ClinVar interpretation had suggested conflicting interpretations of pathogenicity, the collected evidence allowed to classify c.92G>C as uncertain significance, according to ACMG guidelines (table 4),

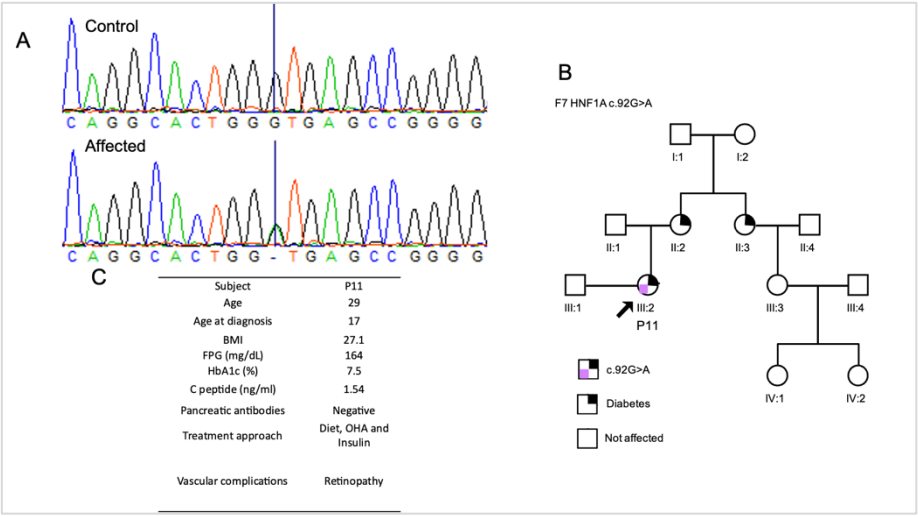

Figure 12 *HNF1A* c.92G>A chromatogram, pedigree and subject data. A: Representation of the variant. B: Pedigree of family number 7. The arrow indicates the proband subject. C: Sum up of feature of subject P11. FPG and HbA1c measurement at enrolment.

Similar to the first one, c.1165T>G (figure 13) was also a missense variant. However, this second variant was detected in exon 6, leading to thymine point substitution by guanine on the first nucleotide position in codon 389 (TTG>GTG). This alteration triggered an amino acid exchange from leucine to valine at the protein level, p.(Leu389Val). It was described in the literature [29] [59] and presented in HGMD, ClinVar, and dbSNP. In silico analysis showed contrasting data, with PROVEAN and SIFT results pointed out to neutral impact while PolyPhen-2 and MutationTaster predicted damaging effect. Moreover, REVEL presented a score of 0.436, which corroborated with divergent in silico data. According to gnomAD, the total MAF was 0.06% and the MAF in the African or African American population of 0.64% (table S5). Data on relatives were not available to ascertain cosegregation. Also, there was no functional evidence for this variation on databases or literature. This variant was evaluated as uncertain significance by ACMG guidelines (table 4). Unfortunately, the lack of evidence did not allow a more accurate classification, as the first variant described above.

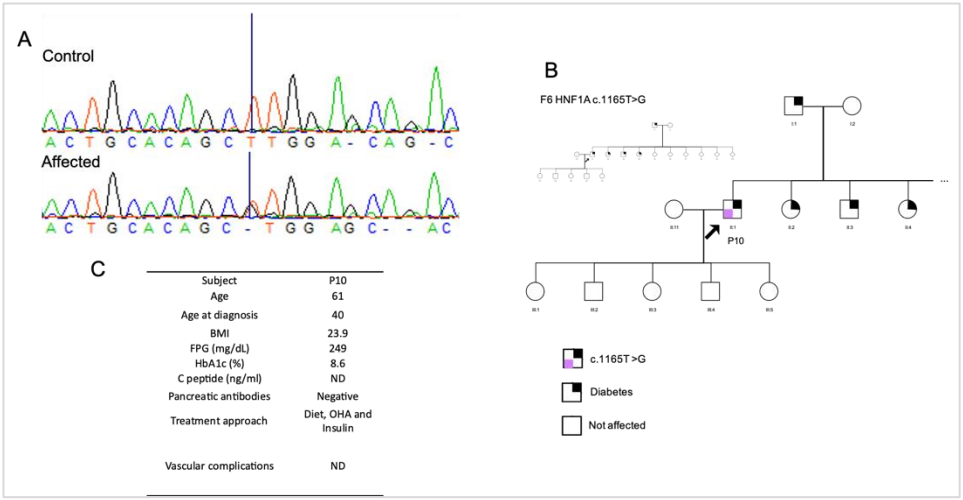

Figure 13 *HNF1A* c.1165T>G chromatogram, pedigree and subject data. A: Representation of control and variant. B: Part of pedigree of family number 6. The arrow indicates the proband subject. Miniature of the whole pedigree. C: Sum up of feature of subject P10. FPG and HbA1c measurement at enrolment. ND meaning not diagnosed.

The next two variants, c.246G>C (figure 14) and c.1392C>T (figure 15), were synonymous variants located in exon 1 and 7, respectively. The c.246G>C variant was a single base substitution of the third position of codon 82 (ACG>ACC) that did not trigger an amino acid

exchange (p.Thr82Thr). Only MutationTaster result presented some effect on the protein leading to unprecise in silico analysis. Moreover, it was not found on any databases conditioning the compilation of other information, such as population frequency. On the other hand, c.1392C>T affected the last nucleotide position of codon 464 (p.Phe464Phe) even though it did not affect protein structure. Curiously, MutationTaster evaluated it as disease causing even though the remaining in silico programs had not agreed with that evaluation. No population frequency was available in gnomAD. Also, the assessment of cosegregation data was not possible, as well no functional studies had been carried out. Despite its absence in reference disease databases for MODY, c.1392C>T was mention in a study about hepatocellular adenoma being present in COSMIC. By ACMG classification both variants are classified as uncertain significance (table 4).

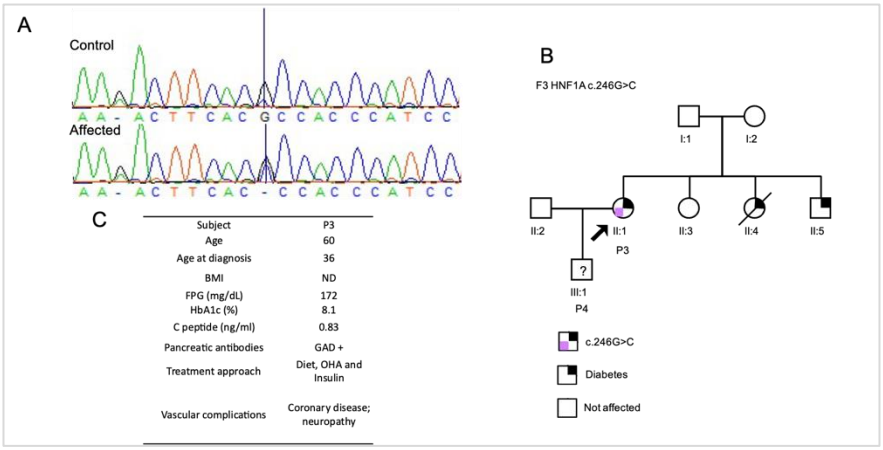

Figure 14 *HNF1A* c.246G>C chromatogram, pedigree and subject data. A: Representation of control and variant. B: Pedigree of family number 3. The arrow indicates the proband subject. Subject P4 showed threshold FPG levels. C: Sum up of feature of subject P3. FPG and HbA1c measurement at enrolment. ND meaning not diagnosed.

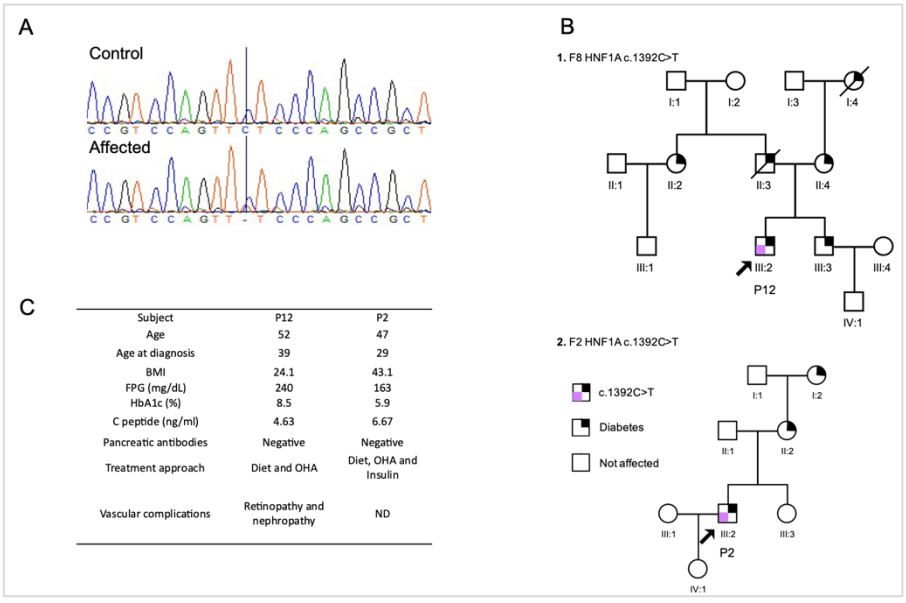

Figure 15 *HNF1A* c.1392C>T chromatogram, pedigrees and subject data. A: Representation of control and variant. B: 1. Pedigree of family number 8. 2. Pedigree of family number 2. The arrow indicates the proband subject. C: Sum up of feature of subjects P12 and P2. FPG and HbA1c measurement at enrolment. ND meaning not diagnosed.

The fifth variant, c.326+3A>G (figure 16), was a point substitution in an intronic site, namely in intron 1, which can affect the splicing of the mRNA. The research on databases of this variant was unsuccessful, and cosegregation could not be evaluated. However, adjacent variants, c.326+1G>A and c.326+4A>G, had already described and classified as pathogenic and

uncertain significance, respectively. Predictions of *in silico* programs for human splice sites variants, namely NNSLIPE and MAXENTSCAN, had a positive result. However, the classification of c.326+3A>G by ACMG guidelines only reached a classification of uncertain significance (table 4).

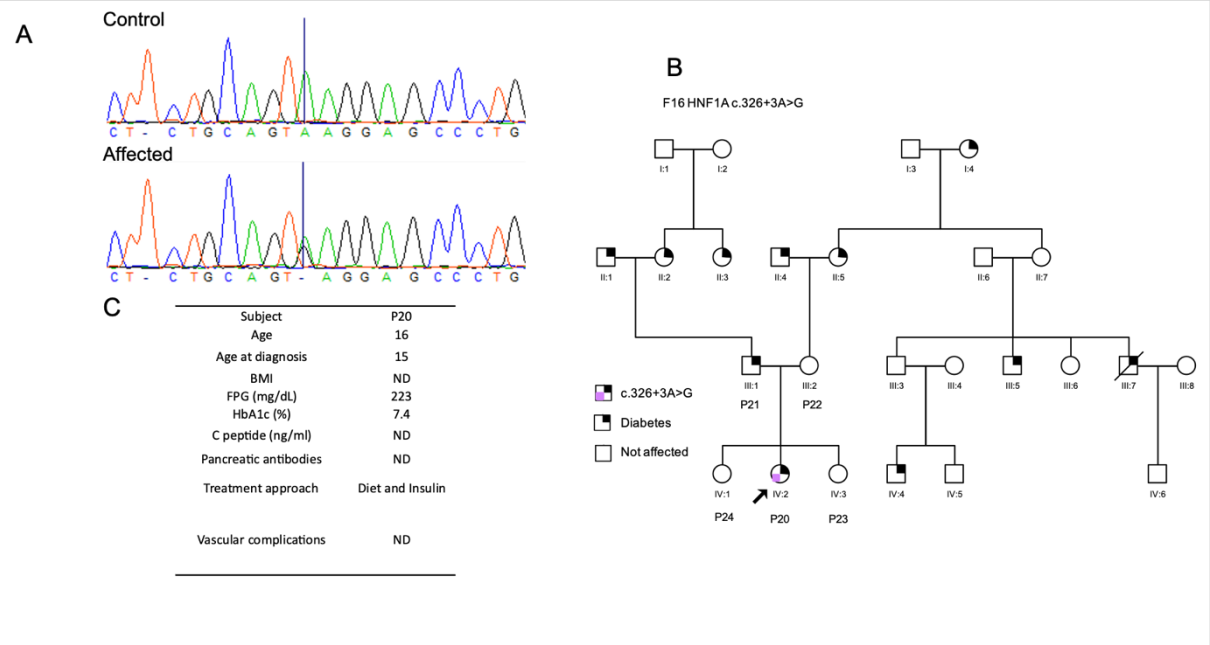

Figure 16 *HNF1A* c.326+3A>G chromatogram, pedigree and subject data. A: Representation of control and variant. B: Pedigree of family number 16. The arrow indicates the proband subject. Subject P21 had retinopathy showing FPG levels of 155 mg/dL. Also, he was on OHA. C: Sum up of feature of subject P20. FPG and HbA1c measurement at enrolment. ND meaning not diagnosed.

MLPA

In addition to Sanger sequencing, all probands were subjected to the MLPA assay. However, no CNV was detected among the 19 subjects.

ACMG classification

All detected variants were classified according to ACMG. Nine were benign and seven variants were classified as uncertain significance. In table 2 are represented the given criteria which justified the final classification.

Table 4 ACMG classification and attributed criteria. ACMG criteria attributed for each detected variant.

Gene	Transcript alteration	Protein alteration	ACMG criteria	ACMG classification
GCK	c.-527C>T	p.(?)	BS1	Uncertain significance
	c.-516G>A	p.(?)	BA1	Benign
	c.129C>T	p.(=)	PM2; PP4; BP7	Uncertain significance
HNF1A	c.51C>G	p.(=)	BA1; BP4; BP7	Benign
	c.79A>C	p.(Ile27Leu)	BA1; BP4	Benign
	c.92G>A	p.(Gly31Asp)	PP2; BS1	Uncertain significance
	c.246G>C	p.(=)	PM2; PP4; BP7	Uncertain significance
	c.326+3A>G	p.(?)	PM2; PP3; PP4	Uncertain significance
	c.327-42G>A	p.(=)	BA1; BP4	Benign
	c.864G>C	p.(=)	BA1; BP4; BP7	Benign
	c.1165T>G	p.(Leu389Val)	PP2; BS1	Uncertain significance
	c.1375C>T	p.(=)	BA1; BP4; BP7	Benign
	c.1392C>T	p.(=)	PM2; PP4; BP7	Uncertain significance
	c.1460G>A	p.(Ser487Asn)	BA1; BP4	Benign
	c.1501+7G>A	p.(?)	BA1; BP4	Benign
	c.1545G>A	p.(=)	BA1; BP4; BP7	Benign

Overall results

Seven patients have variants of unknown significance that with the collection of more case level data, can in the future change their classification. Their clinical phenotype and genotype are presented in table 5.

Table 5 Overview uncertain significance variants and patients' phenotype. BMI measurement at diagnosis. FPG and Hb1Ac measurement at enrolment.

Patient	Variants	Gene	ACMG Classification	Age	Age at diagnosis	BMI	FPG (mg/dL)	Hb1Ac (%)	C peptide (ng/mL)	Pancreatic antibodies	Treatment approach	Vascular complications
P2	c.1392C>T	*HNF1A*	Uncertain Significance	47	29	43.1	163	5.9	6.67	Negative	Diet, OHA and Insulin	Retinopathy
P3	c.-527C>T	*GCK*	Uncertain Significance	60	36	Uknown	172	8.1	0.83	GAD+	Diet, OHA and Insulin	Coronay disease; Nephropathy
	c.246G>C	*HNF1A*	Uncertain Significance									
P10	c.1165T>G	*HNF1A*	Uncertain Significance	61	40	23.9	249	8.6	Unknow	Unknow	Diet, OHA and Insulin	Not Diagnosed
P11	c.92G>A	*HNF1A*	Uncertain Significance	29	17	27.1	164	7.5	1.54	Negative	Diet, OHA and Insulin	Retinopathy
P12	c.1392C>T	*HNF1A*	Uncertain Significance	52	39	24.1	240	8.5	Unknow	Unknow	Diet and OHA	Neuropathy; Nephropathy
P14	c.129C>T	*GCK*	Uncertain Significance	69	45	22.1	121	6.5	3.96	Negative	Diet and OHA	Coronay disease; Neuropathy; Nephropathy
P20	c.326+3A>G	*HNF1A*	Uncertain Significance	16	15	Uknown	223	7.4	Unknow	Unknow	Diet and Insulin	Not Diagnosed

Discussion

This study included samples from 19 patients with diabetes who, in collaboration with APDP, were previously selected for genetic diagnosis since they fulfilled MODY diagnostic criteria. Therefore, most of the participants had clinical features for suspecting that a genetic alteration in a MODY gene could be the pathological cause. The overall age of diagnosis was slightly higher than that of the threshold from the MODY criteria. All candidates had a strong family history of diabetes and fasting plasma glucose above the reference threshold (>100 mg/dL). Despite an overwhelming majority of patients being diagnosed before 25 years, diagnosis of diabetes might be later according to MODY subtype, such as HNF1B-MODY phenotypes, which often present a large range of age at diagnosis. Additionally, lacking knowledge about monogenic diabetes may also be why some patients had not been diagnosed earlier. Curiously, one subject presented positive β-cells pancreatic antibodies, which is a feature associated with T1MD. However, this patient cannot be discarded from the genetic testing since the presence of GADA and IA-2 antibodies had already reported in a few MODY phenotypes [61]. BMI was unexpectedly high with a propensity to increase when compared to the diagnosis and at enrolment data. Environmental factors, lifestyle, inappropriate diet and treatment are possible reasons to explain this tendency. On the other hand, a recent study estimated the prevalence of monogenic diabetes in a cohort of overweight and obese adolescents previously diagnosed with T2DM showed 4.5% of the subjects were undiagnosed MODY even though the atypical elevated BMI [62]. C peptide seems to be the most reliable biomarker for distinguishing between MODY and other diabetes types [11]. Only one subject showed values slightly lower than was expected. In the last years, the extensive implementation of genetic testing for monogenic diabetes brings up the overlap of clinical features between the different type of diabetes. Thus, setting up well-defined inclusive MODY criteria remains a challenge.

Regarding vascular complications diabetes-associated, collected data showed a direct correlation between older ages and a higher prevalence of more than one vascular complication. Only four probands had not any of the four vascular complications under study. Indeed, early diagnosis seems to be a crucial factor in managed diabetes and prevent possible future health complications. As expected, microvascular complications were quite common among the subjects. Retinopathy and neuropathy were broadly identified among probands. Otherwise, most of individuals who had nephropathy were diagnosed with diabetes later, as is expected from HNF1B-MODY patients. The onset of microvascular complications of diabetes depends on both the duration and the severity of hyperglycaemia. More than three-quarters of the sample had HbA1c levels higher than reference values at enrolment. Despite some patients presenting values between a well-controlled glycemia range for patients with diabetes, they still were exposed to damaging hyperglycaemia. This becomes highly concerning for the patients whose Hb1Ac levels pointed out poor-controlled diabetes even though they have been on treatment. These patients have an increased risk of developing or aggravating their microvascular disease. Thus, the management of diabetes and treatment approach should be adjusted to prevent severe health complications.

Overall, FPG and HbA1c had a significant decrease from diagnosis to at enrolment. Indeed, all subjects had a therapy implementation which is reflected in this overall reduction. However, three individuals had a higher value of FPG at the second assessment. Most patients presented an FPG higher than two times of threshold reference. These data allow us to suspect that

treatment approaches were not efficient in the majority of cases. Individual features, MODY type and pathological process triggering the disorder must be considered to ensure sound treatment response.

Unfortunately, data on OTTG was not available from all probands. This information could be important to compare the obtained results and literature. Data on other criteria were also not available for every patient. Consequently, the study sample characterisation may have been less accurate, requesting a careful interpretation of results.

Among the nineteen studied families, sixteen different variants were detected. In general, subjects had FPG levels quite elevated to fill the criteria of GCK-MODY. However, most participants had phenotype which allow to suspect of MODY 3. Thus, it was expected higher prevalence of *HNF1A* pathogenic variants in the sample studied [26].

Seven variants were classified as uncertain significance variants. Two of them were missense, two were synonymous and one may affect protein splicing. Most of the collected data was not enough for an accurate classification. The lack of functional studies, discordance in *in silico* analysis programs, lack of co segregation studies and the absence of the variants in disease databases made data gathering a challenge for the remaining variants. However, if more data is collected there is a chance that the variants can change classification to likely pathogenic, at least for two missense variants. Synonymous variants usually do not cause disease since they maintain the same amino acid. Efforts to increase evidence should be made so these patients can reach a final diagnosis.

The first, *GCK* c.129C>T variant, was a synonymous variant in exon 2 of *GCK* detected in subject P14. *In silico* prediction was not consensual. Despite ClinVar interpretation being likely benign, the low MAF (table S5) and lack of information about this variant in the literature were few of the evidence did not allow us to ascertain classification. This patient presented a FGP slightly higher than reference values, a normal Hb1Ac, and persistent fasting C-peptide production evidence. Moreover, subject P14 was on diet and OHA. All these features are according to *GCK* phenotype.

Curiously, *HNF1A* c.246G>C variant and *GCK* c.-527C>T variant were both detected in patient P3. Variant c.246G>C was a synonymous variant in exon 1 of *HNF1A.* Meanwhile, *GCK* c.-527C>T was an upstream transcript variant. This patient has tested positive for GAD antibodies, although fasting C peptide presence at abnormal levels for T1DM. FPG and Hb1Ac were above than criteria range even though the patient was on combined dieting, OHA, and insulin therapy. Higher blood glycaemia levels and age at diagnosis are few factors that match with *HNF1A*-MODY features. Subject P3 was diagnosed during pregnancy, although it was not possible to ascertain how this condition influences pregnancy management and baby birth weight. Current data from subject P4 evidenced FPG and HbA1c between the *GCK*-MODY range. Also, a considerable level of fasting C peptide allied with elevated OTTG levels corroborates the presence of *GCK* variant in this family as a pathogenic cause of diabetes.

The following uncertain significance variant was the *HNF1A* c.92G>A (rs137853247) variant identified in subject P11. It was a missense variant in exon 1 of *HNF1A. In silico* prediction was not consensual. This variant has been previously reported in several countries such as France [63], U.K [64], and Spain [65]. Patient P11 had FPG and Hb1Ac elevated even though she was on all treatments in the study (diet, OHA, and insulin therapy), and fasting C peptide

levels were between the criteria for *HNF1A*-MODY. Unfortunately, no relatives samples were available to ascertain cosegregation. Indeed, the pathogenicity of c.92G>A variant has been a topic of discussion among experts, which remain without a consensual and unambiguous interpretation [35] [64]. Therefore, functional assays, cosegregation data become especially relevant to produce more evidence about it, providing a better interpretation of classification criteria.

HNF1A c.1165C>T (rs115080759) was also a missense variant detected in subject P10. *In silico* analysis was not conclusive with divergent predictions. It had been reported in France and the USA and presented a MAF of 0,08% (table S5). Additionally, this variant has a higher frequency in the African population, which is subject P10 ethnicity. Subject P10 had elevated FPG and Hb1Ac. However, any vascular complications have been diagnosed. Despite ClinVar interpreting this variant as benign/likely benign, obtained data did not allow us to assign enough criteria for a concordant classification.

HNF1A c.326+3A>G was an intronic variant. *In silico* prediction showed strong evidence for affected splicing. This variant was identified in subject P20, who present higher FGP levels even though she was on dietary and insulin therapy. Lastly, c.1392C>T in the *HNF1A* gene was a synonymous variant detected in subjects P2 and P12. Both were on insulin therapy with no sign of better control of their hyperglycaemia. The absence of databases and the lack of data precludes a more complete and substantiate evaluation of these variants.

Almost half of the detected variants were classified as benign. Likewise, these variants are well represented on databases and in the literature, which allows a better and more robust evidence compilation for accurate classification. Moreover, some of these variants were found in more than one proband. The best example of this is *HNF1A* c.1460G>A variant (rs2464196) in exon 7 was present in seven subjects. It was first described by Urhammer SA et al. in 1997 [62], being identified in several cohorts with a MAF of 32,96% (table S5). This value is significantly higher, considering MODY prevalent. Moreover, this variant does not affect protein function, and it was consensual to consider a polymorphism in ClinVar. The c.1460G>A variant must be classified as benign for MODY.

In conclusion, in seven participants have been found seven variants of unknown significance. The other twelve participants no variant causing disease were found. Seven variants had uncertain significance not allowing a conclusive association between genetic information and clinical features. However, relatives genetic testing of all families might be very important to better interpret the variants identified. This analysis becomes fundamental in uncertain significance variants, whose cosegregation data could change their final classification. For example, c.-527C>T variant in *GCK*, c.1165T>G and c.92G>A variants in HNF1A can be re-classified as benign if cosegregation is not verified. Likewise, variant c.326+3A>G in *HNF1A* can be classified as likely pathogenic if co segregation is seen in more than 4 family members or if it is possible to perform a functional assay showing this variant affects the splicing. but the functional study can be very challenging.

Indeed, this study encountered quite a few challenges in diagnosis, interpretation and classification of the detected variants. Insufficient information about MODY genes makes the distinction between phenotype features of MODY and other types of diabetes very complicated. Overlap features allied with yet lack of knowledge from clinicians are a massive barrier in identifying and selecting patients for MODY genetic testing. Testing more gene candidates

seems to be an excellent way to improve genotype-phenotype correlation results. Investing in advanced sequence techniques, such as NGS, can provide faster and more comprehensive genetic test allowing for a higher positive rate leading to more patients with a confirmed MODY diagnosis. Implementation of these sequencing approaches is becoming more feasible and can include a high range of genes with an excellent cost-effective. Additionally, an increment of genetic testing can provide a better prevalence of MODY, and increase the characterization of clinical features of MODY patients in the Portuguese population.

On the other hand, further studies should emphasise on ascertain the biological and functional impact of variants. In fact, functional studies are a less explored field even though they may significantly contribute to variants classification and a better understanding of MODY phenotypes. Likewise, the collaboration of different MODY research laboratories has an essential role in feeding databases with data from detected variants and defining specific criteria classification specific for MODY. Lastly, produce more robust evidence from the genetic background of MODY could improve healthcare intervention in diagnosis and management. Thus, providing personalised treatment approaches according to individuals features may prevent or attenuate diabetes-associated health complications, which means a significant improvement in life quality.

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Supplementary material

1. Questionnaire

Sampling conditions explanation and patients' data collected from the following questionnaire.

REPÚBLICA PORTUGUESA
SAÚDE

SNS SERVIÇO NACIONAL DE SAÚDE

Instituto Nacional de Saúde Doutor Ricardo Jorge

Estudo molecular da diabetes monogénica (diabetes tipo MODY e neonatal)

Adaptado de "The MODY Project. Molecular studies of MODY-type diabetes" e "Molecular genetic analysis of the GCK, HNF1A and HNF4A genes"

Número do processo: _____
Número da família: _____
(A preencher pelo INSA)

Identificação: Caso índex ☐ Familiar ☐

Nome do caso índex: _____

Parentesco: _____

Nome completo: _____

Morada: _____

Telefone: _____ Número utente de saúde:_____

Data de nascimento: _____ Estado civil: _____

Naturalidade: _____ Sexo: Masculino ☐ Feminino ☐

Origem étnica: Caucasiano ☐ ; Asiático ☐ ; Africano ☐ ; Outro ☐ Qual?_____

Número hospitalar do caso-índex:_____

Critérios para diagnóstico:

Premissas necessárias para o diagnóstico de diabetes tipo MODY:

- ☐ Diagnóstico efetuado antes dos 25 anos de idade em, pelo menos, um membro da família;
- ☐ Transmissão autossómica dominante com, pelo menos, 3 gerações atingidas pela diabetes (Nota: os membros da família podem ter sido mal diagnosticados com DM tipo 1 ou tipo 2);
- ☐ Capacidade de controlo da diabetes sem recurso à insulinoterapia (e sem desenvolver cetose) durante um período de, pelo menos, 2 anos ou níveis significativos de péptido C.

Suspeita de possível diabetes tipo MODY:

- ☐ Características inconsistentes com outros tipos de diabetes: sem obesidade significativa, ausência de acantose nigricans ou evidência de resistência à insulina em DM tipo 2 de início precoce e com história familiar de diabetes, e ausência de auto-anticorpos pancreáticos;
- ☐ Necessidade reduzida de recorrer à insulina; sensibilidade marcada a sulfonilureias;
- ☐ DM gestacional em grávida com hiperglicemia moderada em rastreio pré-natal.

Informação acerca da colheita e envio das amostras (ver Anexo Informativo)

Colheita das amostras:

- ☐ Devem ser colhidos 6 ml (2 x 3 ml) de sangue em tubo de EDTA
- ☐ Sempre que possível, enviar o resultado da prova de tolerância à glicose oral (PTGO)

Pág. 1 de 3

Identificação do médico assistente:

Nome:_____

Morada:_____ Hospital:_____

E-mail:_____Telefone do médico assistente:_____

História clínica: (necessária para análise)

Peso à nascença: ___,_____ kg Gestação (semanas): _____

Idade de diagnóstico: _____anos IMC: _____

Dados aquando do diagnóstico:

Glicémia: _____ mg/dl (data: __ /__ /____) PTGO(2horas): _____mg/dl(data:__/__/____)

HbA1c:_____% (data:__/__/____) Péptido-C:_____ ng/ml (data:__/__/____)

GAD/ICA: _____(data:__/__/____) Peso: _____Kg Altura: _____m

Dados da última consulta:

Glicémia: _____ mg/dl (data: __ /__ /____) PTGO(2horas): _____mg/dl(data:__/__/____)

HbA1c:_____% (data:__/__/____) Péptido-C:_____ ng/ml (data:__/__/____)

GAD/ICA: _____(data:__/__/____) Peso: _____Kg Altura: _____m

Dieta: Sim ☐ Não ☐ Insulina: Sim ☐ Não ☐ ADO: Sim ☐ Não ☐

Outra terapêutica: _____

Complicações:

Retinopatia: Sim ☐ Não ☐ Doença coronária: Sim ☐ Não ☐

Nefropatia: Sim ☐ Não ☐ Polineuropatia: Sim ☐ Não ☐

História Familiar do caso-índex: (Preencher com SIM e NÃO quando os valores não são conhecidos)

Parentesco	**DN**	**Glicémia** (mg/dl)	**PTGO** (2h) (mg/dl)	**Peso (Kg)**	**Altura (m)**	**Diabético?**	**Idade diagnóstico**	**Tratamento**
Pai Nome:								
Mãe Nome:								
Irmão M/F Nome:								
Irmão M/F Nome:								
Filho M/F Nome:								
Filho M/F Nome:								
Cônjuge Nome:								
Outro: Nome:								
Outro: Nome:								

História familiar de: Doença renal? _____ Surdez? _____

Árvore genealógica:

Obs.:_____
_____

O formulário clínico deve ser sempre acompanhado pela DECLARAÇÃO DE CONSENTIMENTO INFORMADO E ESCLARECIDO (CE-IM04)

Estudo molecular da diabetes monogénica (diabetes tipo MODY e neonatal)

Adaptado de "The MODY Project. Molecular studies of MODY-type diabetes" e "Molecular genetic analysis of the GCK, HNF1A and HNF4A genes"

Informação sobre a colheita e envio de amostras

Colheita das amostras:

- Devem ser colhidos 6 ml (2 x 3 ml) de sangue em tubo de EDTA
- Sempre que possível, enviar o resultado da prova de tolerância à glicose oral (PTGO)

Envio das amostras:

Estas amostras devem ser enviadas, juntamente com a documentação, em **correio azul** em envelope almofadado ou numa caixa, bem envolvidas em algodão, **acompanhadas pelo formulário devidamente preenchido**. O tempo máximo entre a colheita das amostras e a sua análise não deverá ultrapassar os **dois dias**, o que implica que a amostra seja enviada logo após a colheita. Esta condição é de extrema importância para este estudo. Qualquer dúvida contactar a Doutora Mafalda Bourbon (217508126) ou Doutor Paulo Dario (217508130).

Também é possível realizar a colheita de sangue no INSA, mediante marcação prévia para os números 217508136/30 (Dra Margarida Vaz, Doutora Mafalda Bourbon, Doutor Paulo Dario).

O formulário clínico deve ser sempre acompanhado pela DECLARAÇÃO DE CONSENTIMENTO INFORMADO E ESCLARECIDO (CE-IM04)

ANEXO INFORMATIVO

2. Genes sequence

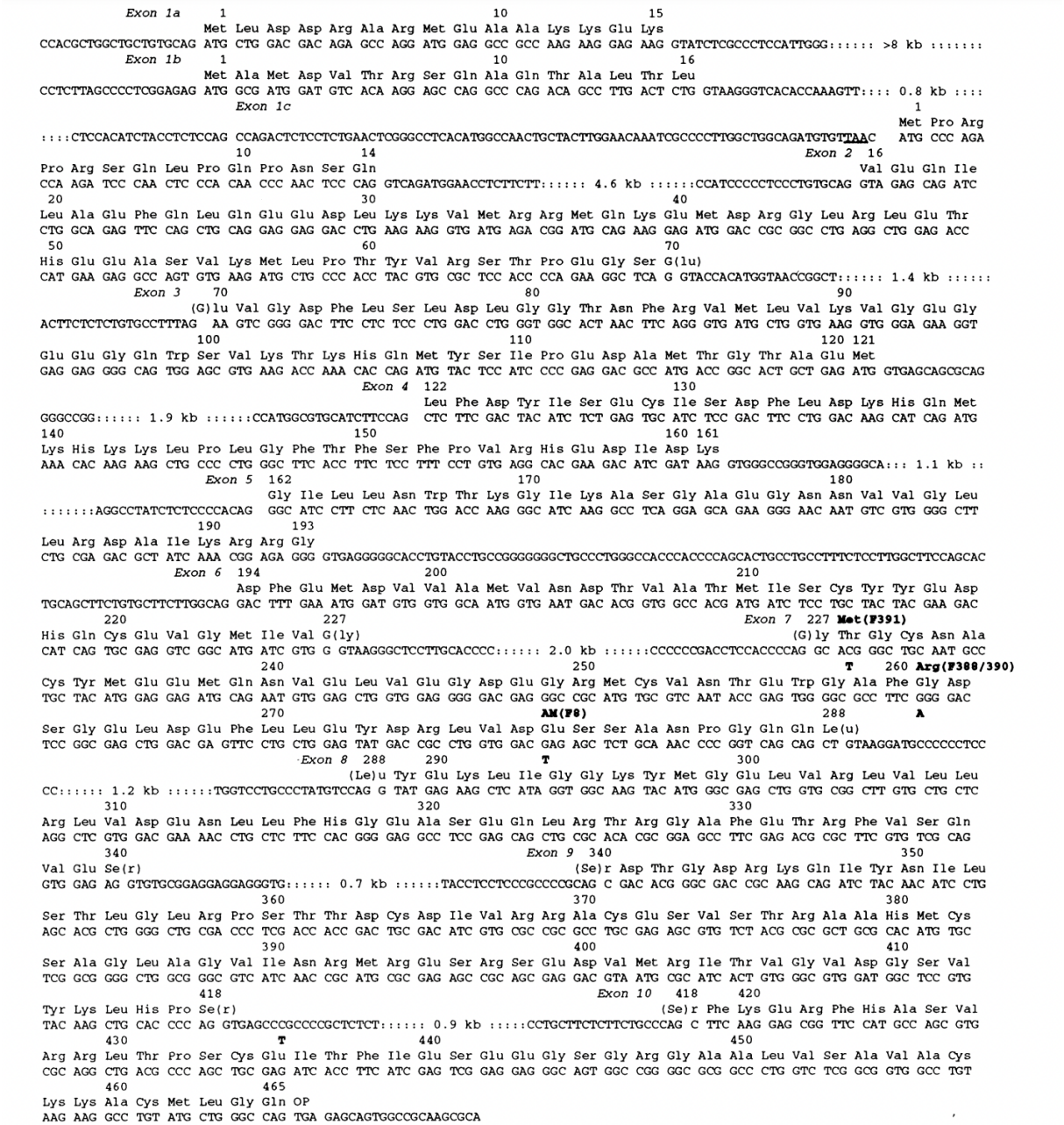


Figure S1 Partial sequence of the human glucokinase gene. Nucleotide and predicted amino acid sequences are shown. The number of the amino acid at the beginning and the end of each exon is noted. Approximate sizes of the introns are indicated. The mutations in exon 7 and the polymorphism in intron 9 are shown in boldface type. Note that the minor liver glucose transcript liver includes both exons lb and 1c. However, translation initiated at the ATG/Met-l in exon lb would terminate at the stop codon (underlined) immediately upstream of Met-i in exon 1c. Similarly, translation beginning at the ATG upstream of Met-1 in exon 1c terminates in exon 2. The frequencies of the C and T alleles of the polymorphism in intron 9 in a group of 30 unrelated nondiabetic Caucasians were 0.80 and 0.20, respectively. Adapted from [36].

3. Primer sequences and PCR standard conditions

Table S1 Primer sequence data. Gene, genomic location, primer size (bp), primer sequence, orientation (F – forward; R – reverse), amplicon size (bp), annealing temperature (ºC), reagents kit used, $MgCl_2$ volume for one reaction, PCR cycle numbers. * Applied Biosystems reagents $MgCl_2$ final concentration of 1.5 mM.

Gene	Genomic location	Primer size (bp)	Primer sequence (5'-3')	Primer type	Amplicon size (bp)	Anneling temperature (ºC)	Reagents Kit	Vol $MgCl_2$ (µL): 1x	$MgCl_2$ final concentration	PCR cycle numbers
GCK	Promotor	20	CCTGTCCAGCTTTGGACTCT	F	598	61	Bioline	0.5	1 mM	38
		20	GTGACCTGGGGACAGCTTTT	R						
	Exon 1	20	TGCATGGCAGCTCTAATGAC	F	754	62		0.75	1.5 mM	38
		20	CCTTCTCAAAGAGCCTGTGC	R						
	Exon 2	18	GTGTGCAGATGCCTGGTG	F	346	62		0.5	1 mM	38
		20	CTGGCTGTGAGTCTGGGAGT	R						
	Exon 3	20	CCTTCCCTCCTCCTCTTTGT	F	364	61		0.75	1.5 mM	38
		19	CCACCCCTGGTAGACAGGT	R						
	Exon 4	20	CGGAAGAGGAGAGGGAAACT	F	382	61		0.75	1.5 mM	38
		20	CCAGATCTCCCTTCTGAGCA	R						
	Exon 5 and 6	20	CTGCTCTGAGCCTGTTTCCT	F	565	62		0.5		38
		20	ACCAGGCTCTGCTCTGACAT	R						
	Exon 7	20	CCATTGTTCCAGACAAAGCA	F	400	61		0.75	1.5 mM	38
		20	CAAGCCCATTATCTGCAATG	R						
	Exon 8	20	CCTCGTGCCTGCTGATGTAA	F	290	60		1		42
		20	GTCGCCCTGAGACCAAGTCT	R						
	Exon 9	18	GCTCAGCGAGGGAAAGAG	F	482	61		0.75	1.5 mM	38
		20	GGGGGACGAGAAGAGGACTA	R						
	Exon 10a	19	GTCCCCCTTGGCCTAGATT	F	698	62		0.5		38
		20	AGGTCTGGTCAAGCTGTTGG	R						
HNF1A	Promotor	20	CTACAGGGAGGCCTGGTGTC	F	585	61		0.5		38
		20	CGGCAGACACAAACCAAACT	R						
	Exon 1	20	AGTCCCTTCGCTAAGCACAC	F	671	61		0.75	1.5 mM	38
		19	GGCTCGTTAGGAGCTGAGG	R						
	Exon 2	20	CCCTGTGTCCTGGCATAAAT	F	489	59		0.75	1.5 mM	38
		20	TGTGTAATGGGGATGGTGAA	R						
	Exon 3	20	GCATGTGTGCTGTGTGTTTG	F	491	61		0.75	1.5 mM	38
		20	GCCAGGCTAAGCCAATATCA	R						
	Exon 4	20	CAGATCTGCCAGCCTCAAAC	F	480	58		0.75	1.5 mM	35
		20	CATGAATGGAATGGAACCAA	R						
	Exon 5 and 6	20	GCCTAAGCAAACCAATGGAG	F	655	61		0.5		38
		20	CTCTTCCAGCTCCTGGATTC	R						
	Exon 7	20	CTCTGGGAAGGAGAGGTGGT	F	397	61		0.75	1.5 mM	38
		20	GTCCCAGAGACACATGCAGA	R						
	Exon 8 and 9	20	AGTCTTGAGGCCTGGGACTA	F	558	61		0.5		38
		19	CTTCCTCACAGCAGCCCTA	R						

	Exon 10a	19	GGTGTGACTTTGGGGTTCC	F	850	61		0.5		38
		20	CAGAGTAGCCACCCAGGAAA	R						
HNF1B	Promotor	21	CATGAACCCCGAAGAGTGGTG	F	423	58	Applied Biosystems	*	1.5mM	35
		20	GCCTCCAGACACCTGTTACT	R				*		
	Exon 1a	22	CTGGATTTGGGGTTTGCTTGTG	F	352	61		*		38
		20	TCATAGTCGTCGCCGTCCTC	R				*		
	Exon 1b	21	CATACTCTCACCAACGGCCAC	F	258	62		*		37
		19	AAACGGGCTTGGCGAGTGT	R				*		
	Exon 2	20	CTCCCACTAGTACCCTAACC	F	291	60		*		38
		22	GAGAGGGCAAAGGTCACTTCAG	R				*		
	Exon 3	22	AGTGAAGGCTACAGACCCTATC	F	365	60		*		38
		21	TTCCTGGGTCTGTGTACTTGC	R				*		
	Exon 4	21	TGTGTTTTGGGCCAAGCACCA	F	381	60		*		38
		20	AACCAGATAAGATCCGTGGC	R				*		
	Exon 5	20	TGCCGAGTCATTGTTCCAGG	F	276	60		*		38
		22	CCTCTTATCTTATCAGCTCCAG	R				*		
	Exon 6	22	CTGCTCTTTGTGGTCCAAGTCC	F	288	58		*		35
		21	GAGTTTGAAGGAGACCTACAG	R				*		
	Exon 7	21	ATCCACCTCTCCTTATCCCAG	F	340	58		*		35
		21	ACTTCCGAGAAAGTTCAGACC	R				*		
	Exon 8	21	TTTGCCTGTGTATGCACCTTG	F	257	58		*		35
		20	GCCGAGTCCATGCTTGCCAC	R				*		
	Exon 9	20	CTTTGCTGGTTGAGTTGGGC	F	208	58		*		35
		21	TTCCATGACAGCTGCCCAGAG	R				*		

Table S2 Bioline reagents kit Standard PCR mix and cycling program. Annealing temperature, $MgCl_2$ volume, and cycle number were adjusted according to each fragment (see table S1 of supplementary material). Each PCR reaction was added 1 μL of DNA sample at 100-400 ng/μL. [a] $MgCl_2$ concentration was according table S1 adjusting H_2O volume to get the total volume for one reaction.

Bioline reagents kit (A)					
Standard PCR cycling program			Standard PCR Mix for one reaction		
Temperature (ºC)	Time		Reagent	Vol (μL)	Final Concentration
95	5 min		H_2O	18.375	-
94	45 sec	38 cycles	100 mM dNTPs mix	0.25	1 mM
Annealing	45 sec		10x NH_4 Reaction Buffer	2.5	1x
72	1 min		50 mM $MgCl_2$ Solution	0.75[a]	1.5 mM
72	7 min		10μM Primer F	1	0.2 μM
4	Pause		10 μM Primer R	1	0.2 μM
			BIOTAQ (5 U/ μL)	0.125	0.625 U/ 25 μL reaction
			DNA sample	1	-
			Volume final	25	

Table S3 Applied Biosystems reagents kit Standard PCR mix and cycling program. Annealing temperature and cycle number were adjusted according to each fragment (see table 1 of supplementary material). Each PCR reaction was added 1 μL of DNA sample at 100-400 ng/μL.

Applied Biosystems reagents kit (B)					
PCR cycling program			Standard PCR Mix for one reaction		
Temperature (ºC)	Time		Reagents	Vol (μL)	Final Concentration
95	5 min		10x PCR Buffer AB	5	1x
95	30 seg		H_2O	41.4	-
Annealing	30 seg	35 cycles	10 mM dNTPs mix	0.4	0.08 mM
72	1 min		20 μM Primer F	1	0.4 μM
72	5 min		20 μM Primer R	1	0.4 μM
4	Pause		AmpliTaq (5 U/ μL)	0.2	1 U/ 50 μL reaction
			DNA sample	1	-
			Volume final	50	

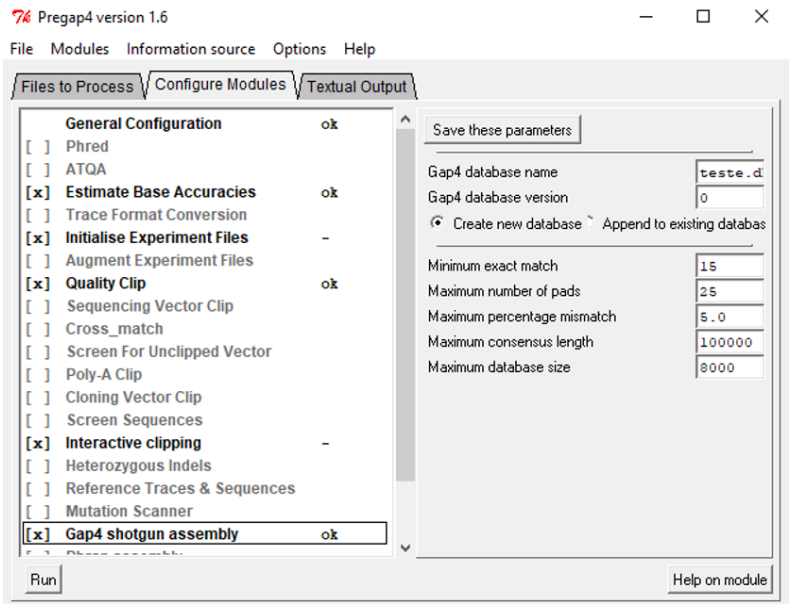
4. Sanger sequencing mix and cycling program

Table S4 Sequencing mix and cycling program. Typical sequencing mix and cycling program. Primer forward is used for most fragments. Exon 6 and exon 8 of *HNF1B* used primer reverse.

Sequencing cycling program			Standard Sequencing Mix for one reaction	
Temperature (ºC)	Time		Reagents	Vol (μL)
96	30 seg		Big Dye Buffer (10x)	0.5
96	10 seg	30 cycles	BigDye	1
58	5 seg		H_2O	6.5
60	4 min		Primer (ρmol/μL)	1
4	Pause		PCR product	1
			Volume Final	10

5. Sequence analysis

Sequence chromatogram files were assembled by Pregap4 according to the following default configuration.



Afterwards, Gap4 analysis was performed by aligning target sequence chromatograms against reference sequence and unrelated subjects sequence chromatogram. The variant screening was made manually.

6. Online Tools

- Mutalyzer:
https://mutalyzer.nl/name-checker
- MutationTaster:
http://www.mutationtaster.org/
- PolyPhen-2:
http://genetics.bwh.harvard.edu/pph2/
- PROVEAN/SIFT:
http://provean.jcvi.org/genome_submit_2.php?species=human
- REVEL:
https://sites.google.com/site/revelgenomics/
- NNSLIPE:
https://www.fruitfly.org/seq_tools/splice.html
- MAXENTSCAN:
http://hollywood.mit.edu/burgelab/maxent/Xmaxentscan_scoreseq.html

7. Interpretation from ACMG criteria

Table S5 PP3 and BP4 computational (in silico) data. Interpretation from in silico programs prediction. MutationTaster gives "polymorphism" (*i.e* benign) or "disease causing" (*i.e* pathogenic). PolyPhen-2 gives "benign" or "possibly damaging" (*i.e* pathogenic). PROVEAN gives "neutral" (*i.e* benign) or "deleterious" (*i.e* pathogenic). SIFT gives "tolerated" (*i.e* benign) or "damaging" (*i.e* pathogenic). Direct correlation between REVEL score and disease variant probability. Rate variant/wild type lower than 0,4 in NNSPLICE and MAXENTSCAN affects mRNA splicing.

Criteria Attributed	Programs	In silico Prediction
	Missense • MutationTaster.	1. All in silico programs agree on pathogenic impact prediction;

PP3	• PolyPhen-2 • PROVEAN • SIFT • REVEL	2. Four in silico programs agree on pathogenic impact prediction; 3. Three in silico programs agree on pathogenic impact prediction and REVEL score is higher than 0.65.
	Splicing • NNSPLICE • MAXENTSCAN	All in silico programs agree on pathogenic impact prediction
BP4	Missense • MutationTaster. • PolyPhen-2 • PROVEAN • SIFT • REVEL	All in silico programs agree on bening impact prediction
	Splicing • NNSPLICE • MAXENTSCAN	All in silico programs agree on bening impact prediction

To attribute PM5 criteria was consider novels missense amino acid change at the same position of established pathogenic or likely pathogenic missense variants.

8. Detected Variants

Table S6 Detected sequence variants. List of all detected sequence variants among *GCK*, *HNF1A* and *HNF1B* in the studied sample. NA – Not applicable; NF – Not Found

Transcript alteration	Location	Chromosomal alteration	Genomic description	Protein alteration	Protein domain	alteration type - structural	alteration type - functional	ClinVar ID	ClinVar interpretation	dbSNP	MAF Total % (gnomAD)	MAF popmax % (gnomAD)	popmax	ACMG classification	First described reference
GCK (NG_008847.2; NM_000162.5; NP_000153.1)															
c.-527C>T	Upstream Transcript Variant	g.44229079G>A	g.13691C>T	p.(?)	NA	point substitution	Upstream Transcript Variant	NF	NA	rs112257899	0,20%	0,69%	African/African American	Uncertain significance	NA
c.-516G>A	Upstream Transcript Variant	g.44229068C>T	g.13702G>A	p.(?)	NA	point substitution	Upstream Transcript Variant	NF	NA	rs1799884	16%	20%	Latino/Admixed American	Benign	[67]
c.129C>T	Exon 2	g.44192979G>A	g.49791C>T	p.(=)	small-domain	point substitution	synonymous	255400	Likely benign	rs760912915	0,002%	0,005%	East Asian	Uncertain significance	NA
HNF1A (NG_011731.2; NM_000545.8; NP_000536.6)															
c.51C>G	Exon 1	g.121416622C>G	g.5074C>G	p.(=)	dimerization domain	point substitution	synonymous	129234	Benign	rs1169289	46,45%	58,20%	Ashkenazi Jewish	Benign	[69]
c.79A>C	Exon 1	g.121416650A>C	g.5102A>C	p.(Ile27Leu)	dimerization domain	point substitution	missense	14937	Benign	rs1169288	34,79%	46,59%	Ashkenazi Jewish	Benign	[69]
c.92G>A	Exon 1	g.121416663G>A	g.5115G>A	p.(Gly31Asp)	dimerization domain	point substitution	missense	14948	Conflicting interpretations of pathogenicity	rs137853247	0,08%	0,16%	Latino/Admixed American	Uncertain significance	[70]
c.246G>C	Exon 1	g.121416817G>C	c.5269G>C	p.(=)	DNA-binding domain	point substitution	synonymous	NF	NA	NF	NF	NF	NF	Uncertain significance	NA
c.326+3A>G	Intron 1	g.121416900A>G	g.5352A>G	p.(?)	NA	point substitution	splicing?	NF	NA	NF	NF	NF	NF	Uncertain significance	NA
c.327-42G>A	Intron 2	g.121426594G>A	g.15046G>A	p.(=)	NA	point substitution	splicing?	673535	Benign	rs1169294	34,20%	45,36%	Ashkenazi Jewish	Benign	[71]
c.864G>C	Exon 4	g.121432117G>C	g.20569G>C	p.(=)	transactivation domain	point substitution	synonymous	129236	Benign	rs56348580	26,37%	44,19%	Latino/Admixed American	Benign	[69]
c.1165T>G	Exon 6	g.121434401T>G	g.22853T>G	p.(Leu389Val)	transactivation domain	point substitution	missense	134511	Benign/Likely benign	rs115080759	0,06%	0,64%	African/African American	Uncertain significance	[29]
c.1375C>T	Exon 7	g.121435342C>T	g.23794C>T	p.(=)	transactivation domain	point substitution	synonymous	129229	Benign	rs2259820	33,02%	48,82%	East Asian	Benign	[62]
c.1392C>T	Exon 7	g.121435359C>T	g.23811C>T	p.(=)	transactivation domain	point substitution	synonymous	NF	NA	NF	NF	NF	NA	Uncertain significance	[68]
c.1460G>A	Exon 7	g.121435427G>A	g.23879G>A	p.(Ser487Asn)	transactivation domain	point substitution	synonymous	129230	Benign	rs2464196	32,96%	48,59%	East Asian	Benign	[62]
c.1501+7G>A	Intron 7	g.121435475G>A	g.23927G>A	p.(?)	transactivation domain	point substitution	splicing?	129231	Benign	rs2464195	38,00%	52,06%	South Asia	Benign	[62]
c.1545G>A	Exon 8	g.121437114G>A	g.25566G>A	p.(=)	transactivation domain	point substitution	synonymous	129232	Benign	rs55834942	13,98%	22,28%	European (Finnish)	Benign	[69]