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14-fold increased prevalence of rare glucokinase gene variant carriers in unselected Danish patients with newly diagnosed type 2 diabetes

Anette P. Gjesing [a,*], Line Engelbrechtsen [a,b], Anne Cathrine B. Thuesen [a,c], Christian T. Have [a], Mette Hollensted [a], Niels Grarup [a], Allan Linneberg [d,e,f], Jens Steen Nielsen [g], Lotte B. Christensen [h], Reimar W. Thomsen [h], Kristoffer E. Johansson [i], Matteo Cagiada [i], Sarah Gersing [i], Rasmus Hartmann-Petersen [i], Kresten Lindorff-Larsen [i], Allan Vaag [c], Henrik T. Sørensen [h], Ivan Brandslund [j,k], Henning Beck-Nielsen [l], Oluf Pedersen [a], Jørgen Rungby [g,m], Torben Hansen [a,*]

[a] Novo Nordisk Foundation Center for Basic Metabolic Research, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[b] Department of Gynecology and Obstetrics, Herlev Hospital, Denmark
[c] Steno Diabetes Center Copenhagen, Gentofte, Denmark
[d] Center for Clinical Research and Prevention, Bispebjerg and Frederiksberg Hospital, Copenhagen, Denmark
[e] Department of Clinical Experimental Research, Rigshospitalet, Glostrup, Denmark
[f] Department of Clinical Medicine, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark
[g] The Danish Centre for Strategic Research in Type 2 Diabetes (DD2), Steno Diabetes Center Odense, Odense University Hospital, Odense, Denmark
[h] Department of Clinical Epidemiology, Aarhus University Hospital and Department of Clinical Medicine, Aarhus University, Aarhus, Denmark
[i] The Linderstrøm-Lang Centre for Protein Science, Department of Biology, University of Copenhagen, Ole Maaløes Vej 5, DK-2200 Copenhagen, Denmark
[j] Department of Clinical Biochemistry, Hospital Lillebaelt, Vejle, Denmark
[k] Institute of Regional Health Research, University of Southern Denmark, Odense, Denmark
[l] Diabetes Research Centre, Department of Endocrinology, Centre for Individualized Medicine in Arterial Diseases, Odense University Hospital, Odense, Denmark
[m] Department of Endocrinology and Copenhagen Center for Translational Research, Bispebjerg Hospital, University of Copenhagen, Copenhagen, Denmark

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ABSTRACT

Aims: Rare variants in the glucokinase gene (*GCK*) cause Maturity-Onset Diabetes of the Young (MODY2/*GCK*-MODY). We investigated the prevalence of *GCK* variants, phenotypic characteristics, micro- and macrovascular disease at baseline and follow-up, and treatment among individuals with and without pathogenic *GCK* variants.
Methods: This is a cross-sectional study in a population-based cohort of 5,433 individuals without diabetes (Inter99 cohort) and in 2,855 patients with a new clinical diagnosis of type 2 diabetes (DD2 cohort) with sequencing of *GCK*. Phenotypic characteristics, presence of micro- and macrovascular disease and treatment information were available for patients in the DD2 cohort at baseline and after an average follow-up of 7.4 years.
Results: Twenty-two carriers of potentially deleterious *GCK* variants were found among patients with type 2 diabetes compared to three among 5,433 nondiabetic individuals [OR = 14.1 (95 % CI 4.2; 47.0), $p = 8.9*10^{-6}$]. Patients with type 2 diabetes carrying *GCK* variants had significantly lower waist circumference, hip circumference and BMI, compared to non-carriers. Three *GCK* variant carriers with diabetes had microvascular complications during follow-up.
Conclusions: Approximately 0.8% of Danish patients with newly diagnosed type 2 diabetes carry non-synonymous variants in *GCK* and resemble patients with *GCK*-MODY. Glucose-lowering treatment cessation should be considered in this subset of diabetes patients.

1. Introduction

Maturity-Onset Diabetes of the Young (MODY) is a clinical definition of diabetes in patients presenting with autosomal dominant heritance, age-of-diagnosis before 25 years of age, and residual C-peptide production. A large fraction of patients clinically diagnosed with MODY

* Corresponding authors at: Novo Nordisk Foundation Center for Basic Metabolic Research Hansen Group, Blegdamsvej 3B, DK-2200 Copenhagen N, Denmark.
E-mail addresses: anette.gjesing@sund.ku.dk (A.P. Gjesing), torben.hansen@sund.ku.dk (T. Hansen).

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have a known genetic etiology, with variants in the gene encoding glucokinase (*GCK*-MODY*)* being among the most common causes [1–3].

By phosphorylating glucose to glucose-6-phosphate, glucokinase functions as the pancreatic beta-cell glucose sensor, regulating glucose-stimulated insulin secretion [4]. In addition, glucokinase plays a critical role in the hepatic conversion of glucose into glycogen, which is a major contributor to the removal of glucose from the portal vein in the post-prandial state [5]. Moreover, reduced glucokinase activity is also thought to play a key role in the central response to hypoglycaemia [6].

Patients with *GCK*-MODY maintain the capacity to produce insulin. However, the level of glucose necessary to stimulate insulin secretion is increased reflecting a higher set point for glucose to enhance pancreatic insulin secretion. *GCK*-MODY patients also have an elevated hepatic glucose output, and the hormonal counter-regulation is set at higher glucose concentrations [7]. Thus, *GCK*-MODY patients have a slightly increased fasting plasma glucose concentration between 5.5 and 8 mmol/l [8]. Following an oral glucose tolerance test (OGTT), *GCK*-MODY patients only have a minor increase in plasma glucose (often < 3 mmol/l), and<50 % have 2-hour plasma glucose values exceeding the diagnostic threshold for diabetes at 11.1 mmol/l [7]. Therefore, *GCK*-MODY patients are often asymptomatic and diagnosed incidentally, e.g. in connection with pregnancy or if familial diabetes is suspected. *GCK*-MODY is present from birth and glucose levels only slightly deteriorate with age [9]. The penetrance of pathogenic *GCK* variants is complete and there is a homogenous phenotype among affected family members [10]. Glucose-lowering therapy seems ineffective in patients with *GCK*-MODY and consequently, treatment outside of pregnancy is not recommended [11].

A European case-control study sequenced *GCK* in 2,872 nondiabetic control individuals and in 4,016 patients with type 2 diabetes, among whom a third of the patients were diagnosed before age 40 years. Twenty missense variants were identified, representing a prevalence of 0.5 % among patients with type 2 diabetes versus a prevalence of 0.035 % in controls [12]. A second case-control study including 2,178 patients with type 2 diabetes and 4,170 nondiabetic individuals, found that 0.6 % of patients with diabetes were carriers of *GCK* variants classified as pathogenic or likely pathogenic yielding a sevenfold higher prevalence among patients with diabetes [OR of 7.08 (95 % CI: 2.23–27.3)]. The carriers with diabetes were significantly leaner than non-carriers with diabetes. An equal proportion of carriers and non-carriers were treated with glucose-lowering agents [13].

One previous cross-sectional study examined the prevalence of micro- and macrovascular complications in 99 *GCK*-MODY patients with a mean age greater than 50 years, 91 nondiabetic individuals and 83 young-onset diabetes patients. The proportion of non-severe retinopathy was similar or slightly higher among *GCK*-MODY patients compared to healthy controls. However, no severe eye disease was found among *GCK*-MODY patients. Neuropathy and microalbuminuria were rare in patients with *GCK*-MODY (2 % and 1 % of the participants respectively) with risks similar to those found in nondiabetic individuals [9].

The aim of this study was to examine the prevalence and the clinical presentation of *GCK* variants in a nondiabetic population-based cohort and in a cohort of newly diagnosed patients with type 2 diabetes. A novelty of the current study is the investigation of a large cohort of newly diagnosed type 2 diabetes patients with phenotypic characteristics at the time of diagnosis and up to 10 years of complete follow-up for use of medications and development of micro- and macrovascular disease. Additionally, a correct identification of pathogenic variants is essential to accurately assess the clinical consequence of carrying deleterious variants. Thus, pathogenicity classification was related to a cell based functional assay (MAVE) and an *in silico* functionality score (GEMME).

2. Methods and materials

2.1. Subjects

Targeted sequencing was performed in the population-based Inter99 cohort. Glucose tolerance was classified based on a 2-hour OGTT using the 1999 World Health Organisation criteria [14]. Based on this classification, we included 4,413 glucose tolerant individuals and 1,020 prediabetic individuals from the Inter99 cohort, who are collectively referred to as nondiabetic individuals (n = 5,433). In addition, we sequenced 2,855 patients with newly diagnosed type 2 diabetes with available DNA samples from the nationwide Danish DD2 cohort. Phenotypic information was available for 2,824 of these individuals [15]. The DD2 cohort has enrolled patients since 2010 and is ongoing. Patients from the DD2 cohort included in the present study were those recruited until 2015. Patients with newly diagnosed diabetes were either enrolled by their general practitioner or from a hospital clinic, on average between 1 and 1.5 years after their first record of glucose-lowering therapy [16]. All patients were GAD65 antibody-negative and had a fasting serum C-peptide above 150 mmol/l (if available). Clinical characteristics of participants can be found in Table 1.

Informed consent was obtained from all participants. The study design was in accordance with the ethical scientific principles of the Helsinki Declaration II.

2.2. Anthropometrics and biochemistry

All participants in the Inter99 cohort had body weight and height measured wearing light indoor clothes and without shoes. Waist circumference at the umbilical level was measured on subjects in an upright position to the nearest 0.5 cm using a non-extendable linen tape measure according to the WHO recommendation.

In the Inter99 study cohort, a standard 75 g OGTT was performed after a 12-hour overnight fast. Fasting serum insulin levels (excluding des-31,32 and intact proinsulin) were measured using the AutoDELFIA insulin kit (Perkin-Elmer, Wallac, Turku, Finland) and fasting plasma glucose was analysed using a glucose oxidase method (Granutest; Merck, Darmstadt, Germany) [17]. Serum triglycerides and total and high-density lipoprotein (HDL) serum cholesterol were analysed using enzymatic colorimetric methods (GPO-PAP and CHOD-PAP, Roche Molecular Biochemicals, Germany). HbA1c was measured using ion-exchange high performance liquid chromatography (normal reference range: 4.1 %-6.4 %).

For patients from the DD2-cohort, anthropometric measures such as height and weight and biomarkers including HbA1c, lipids and auto-antibodies were extracted from the Danish Diabetes Database for Adults [16]. Laboratory values, such as fasting plasma glucose, fasting plasma

Table 1
Clinical characteristics of study participants.

Trait	Non-diabetic participants (Inter99 cohort, n = 5,433)	Newly diagnosed diabetes patients (DD2 cohort, n = 2,824)
Sex (men/women)	2625/2808	1662/1162
Age at enrolment (years)	45.0 (40.0; 50.1)	61.9 (53.3; 68.2)
Age at diagnosis (years)	NA	60.3 (51.7; 66.9)
BMI (kg/m^2)	25.3 (23.0; 28.2)	30.6 (27.1; 34.6)
Waist/hip ratio	1.0 (0.9; 1.0)	0.85 (0.78; 0.91)
Fasting plasma glucose (mmol/l)	5.4 (5.1; 5.7)	7.1 (6.4; 8.1)
Fasting serum C-peptide (pmol/l)	518 (407; 678)	1138 (862; 1497)
Triglycerides (mmol/l)	1.0 (0.8; 1.5)	1.6 (1.1; 2.4)
Total cholesterol (mmol/l)	5.4 (4.8; 6.1)	4.4 (3.7; 5.1)

Data are presented as medians and interquartile ranges. BMI: Body mass index.

C-peptide and C-reactive protein (CRP) were measured directly using biobank samples. C-peptide levels were measured using the ADVIA Centaur C-Peptide assay (Siemens Healthcare Diagnostics ltd, Frimley, Camberley, UK) and fasting plasma glucose levels were analysed using an enzymatic hexokinase method (*gluco*-quant Glucose/HK, Roche Diagnostics). The particle-enhanced immunoturbidimetric method using Tina-quant C-reactive Protein Gen.3 (Roche Diagnostics GmbH, Mannheim, Germany) was used to measure CRP, allowing measurement of CRP within the limits of 0.3–350 mg/l [16].

Information on micro- and macrovascular disease was based on a complete hospital inpatient and outpatient contact history starting 10 years before the diabetes diagnosis date and ending on 1st of August 2018. Average follow-up time was 7.4 years after diagnosis. Denmark has a free tax supported health care system [18].

Data were extracted from the Danish National Patient Registry (DNPR), which contains all discharge records from all Danish hospitals since 1977 and from hospital outpatient clinic and emergency visits since 1995 [19]. Microvascular diseases were identified in the DNRP based on the presence of retinopathy, atherosclerotic eye disease, blindness, severe vision impairment, use of retinal photocoagulation therapy, neuropathy, nephropathy, albuminuria and chronic dialysis or renal failure. Macrovascular diseases were extracted based on the presence of ischemic heart disease, atherosclerotic cerebrovascular disease, atherosclerotic peripheral vascular disease, or any operation for macroangiopathy [20–21].

Treatment information was retrieved from the Danish National Health Service Prescription Database [20] using the treatment codes in Supplementary Table 1. Treatment patterns were investigated among patients with newly diagnosed diabetes one year prior to study inclusion and until present day and for the current study, data was included from 1st of January 2004 until 1st of August 2018.

2.3. Targeted resequencing platform

Targeted sequencing was performed using a chip-based customized nucleotide probe designed to capture the coding regions of *GCK*. Methods for DNA extraction, target region capture, and NGS have been extensively described previously [22]. The final captured DNA libraries were sequenced using the Illumina HiSeq2000 Analyzers as PE 90 bp reads following the manufacturer's standard cluster generation and sequencing protocols. All of the variable sites were covered with a minimum depth of 30X with a mean depth of 182X. The variants located in the *GCK* region were annotated according to the NM_000162 transcript. Variants were included in the study if they were nonsense, frameshift or missense variants, or located up to two nucleotides into intron/exon boundaries. All identified variants were verified using Sanger sequencing.

2.4. Pathogenicity of variants

Variants were classified in accordance with the American College of Medical Genetics and Genomics (ACMG) terminology: Benign, likely benign, variants of uncertain significance (VUS), likely pathogenic or pathogenic [23]. If variants were present in the ClinVar database [24] variants were classified according to this, with all variants being classified no later than 2015. If ACMG classification was available in addition to other classifications, the ACMG classification was selected. If variants were not present in ClinVar, variants were classified manually according to ACMG guidelines [23]. In the remainder of the manuscript, variants classified as either likely pathogenic or pathogenic will be denoted as pathogenic, variants classified as either likely benign or benign will be denoted as benign and variants classified as variants of uncertain significance will be denoted as VUS.

2.5. Functionality score

The activity of eight of the identified variants was investigated using the results from a multiplexed assay of variant effects (MAVE), also known as deep mutational scanning, in which nearly all possible variants of human GCK were assayed for their function using a yeast-based complementation assay [25]. The method is described in [25] and the full dataset is available on MaveDB.org under accession number urn: mavedb:00000096-a.

An *in silico* functionality score was calculated for each of the identified missense variants using information from evolutionary sequence conservation. First, HHblits [26] was used to generate a multiple sequence alignment of 1179 GCK homologs with an E-value threshold of 10^{-20}. This was reduced to 1079 homologs by filtering out sequences with more than 50 % gaps. From this, an evolutionary conservation score was calculated using the Global Epistatic Model for predicting Mutational Effects (GEMME) software [27].

2.6. Statistical analyses

Body Mass Index (BMI) was defined as weight in kilograms divided by height in meters squared (kg/m^2). HbA1c stability was calculated as the maximal change in HbA1c from baseline and throughout the study period. The statistical difference in carrier-frequency between patients with diabetes and participants without, was calculated both as the prevalence difference with 95 % confidence intervals (CI) and using Fisher's exact test. Differences in quantitative traits between carriers of *GCK* variants and non-carriers among newly diagnosed type 2 diabetes patients and nondiabetic individuals were analysed using the difference in the means with 95 % (CI) and the Student's *t*-test comparing the means of phenotypic characteristics. The following traits were log-transformed prior to statistical analysis: BMI, CRP, fasting C-peptide, fasting plasma glucose, HbA1c, HDL-cholesterol, low-density lipoprotein (LDL) cholesterol, total cholesterol, triglycerides and maximal change in HbA1c. The mean difference was calculated by subtracting the means from the compared groups. The CI for the mean difference between log transformed traits, is the CI for the ratio between the two variables. A CI for log transformed traits around one signifies that variables are similar, whereas for non-transformed traits a CI around zero signifies that the two variables are similar. In addition a p-value < 0.05 was considered statistically significant.

Statistical analyses were performed using RStudio software (version 3.6.1 and 4.0.2; R Foundation for Statistical Computing, Boston, MA, USA).

The prevalence of micro- and macrovascular diseases was assessed in the 10 year time period prior to type 2 diabetes diagnosis date. Prevalence for disease was expressed in terms of proportions, and 95 % CIs were calculated using the Wilson Score method. The 95 % CI for the crude prevalence ratio was calculated by log-transformation of the prevalence ratio and use of the delta method.

Incidence of micro- and macrovascular complications was assessed from the beginning of the follow-up period which was the date of type 2 diagnosis until 1st of August 2018. Patients with a prevalent diagnosis of a microvascular disease were excluded from the analysis of microvascular complications. The incidence rate was expressed as number of events per 100 patient-years together with the Poisson exact CIs. The CI for the incidence rate ratio was calculated by log-transformation of the incidence rate ratio and using the delta method.

Differences in prescriptions patterns between patients with type 2 diabetes with and without pathogenic *GCK* variants from 1st of January 2004 until 1st of August 2018, was examined using the Wilson score method to estimate the 95 % CI and the z-test for calculating the p-value for equal proportions.

3. Results

Sequencing of *GCK* in 8,288 individuals with different levels of glucose tolerance, identified a total of 24 variants in 29 carriers. The amino acid variant p.Gly72Arg was present with two different underlying nucleotide substitutions (c.214G > C and c.214G > A), thus a total of 21 missense variants, one stop variant and one frameshift variant were found in 29 carriers (Supplementary Table 2).

All identified variants were rare (MAF < 0.1 %) and six of the variants have not previously been described in international reference databases [28] nor in MODY-families [29]. However, the p.Asp4Asn has been described previously as a polymorphism [29] and the p.Cys221Tyr has been seen in a MODY family also carrying a homozygous *HNF1A* variant [30].

3.1. Prevalence of variants

In the Inter99 cohort of nondiabetic carriers, five non-synonymous variants were identified (four carriers were glucose tolerant and one had impaired fasting glycemia) resulting in a prevalence of non-synonymous variants in *GCK* among nondiabetic individuals of 0.092 % (Supplementary Table 2).

Within the DD2 cohort of patients with newly diagnosed diabetes, 24 carriers of non-synonymous *GCK* variants were found resulting in a prevalence of 0.84 % (Supplementary Table 2). One patient carried two *GCK* variants (p.Asp124Asn and p.Glu279Gln).

3.2. Pathogenicity classification of variants

The variants identified were assessed with regard to their pathogenicity based on the ACMG classification. This classification was compared with results from a functional assay of GCK using yeast complementation (MAVE score) for 18 of the variants found in the present study and the GEMME score was calculated for all of the 21 missense variants from the present study.

The MAVE score cut-off for loss of function variants is < 0.6. One ACMG classified pathogenic variant had a MAVE score above 0.6. This was the p.Gly318Arg variant with a score of 1.3. The GEMME score showed complete segregation between pathogenic and non-pathogenic variants with all pathogenic variants having a score below minus two (Supplementary Fig. 1).

3.3. Variants associated with diabetes

In the Inter99 cohort, two nondiabetic individuals were carriers of benign variants and three were carriers of VUS. In DD2, two patients carried benign variants, five carried VUS and 17 carried pathogenic variants (Supplementary Table 2).

In a cross-sectional case-control analysis comparing the prevalence of identified non-synonymous *GCK* variants between nondiabetic persons and patients with newly diagnosed type 2 diabetes, a 14-fold higher prevalence of pathogenic or VUS *GCK* variants was seen in patients with newly diagnosed type 2 diabetes [OR (95 % CI) = 14.1 (4.2–47.0), p = 8.9*10–6] (Supplementary Fig. 1 and Supplementary Table 2). Variants classified as pathogenic were found exclusively in patients with newly diagnosed type 2 diabetes.

3.4. Phenotypic characteristics of patients with newly diagnosed type 2 diabetes carrying GCK variants.

The phenotype of patients with newly diagnosed type 2 diabetes carrying pathogenic *GCK* variants was compared to patients with type 2 diabetes and nondiabetic individuals without *GCK* variants (Fig. 1 and Supplementary Table 3). Carriers and non-carriers with diabetes had a similar age at enrolment (58.1 years in carriers versus 61.9 years in non-carriers, p = 0.2), but there were fewer men among carriers (24 %) versus non-carriers (60 %), p = 0.003. Measures of body composition were significantly different between carriers and non-carriers with diabetes. Waist circumference was lower among carriers compared to non-carriers with diabetes [carriers: 88 cm (IQR: 80–104 cm); non-carriers: 106 cm (IQR: 97–116 cm); p = $1.8*10^{-5}$] with a mean difference of 15.7 cm (95 % CI:8.5–22.8 cm). Also hip circumference [carriers: 100 cm (IQR: 97–106 cm); non-carriers: 108 cm (IQR: 101–116 cm); p = 0.006] and BMI [carriers: 24.3 kg/m^2 (IQR: 22.8–30.8 kg/m^2); non-carriers: 30.6 kg/m^2 (IQR: 27.1–34.6 kg/m^2), p = 0.02] was lower among carriers compared to non-carriers with diabetes with a mean differences of 8.9 cm (95 % CI: 2.6–15.2 cm) and 1.2 (95 % CI: 1.0–1.3), respectively. Waist and hip circumference as well as BMI among carriers with diabetes were comparable to those of nondiabetic individuals (Fig. 1 A-C and Supplementary Table 3).

Measures of glucose metabolism, expressed as fasting plasma glucose and HbA1c, did not differ between carriers of pathogenic variants and non-carriers with diabetes (Fig. 1 D-E and Supplementary Table 3). HbA1c stability was also examined by comparing maximal HbA1c change observed between study enrolment and throughout follow-up. A significantly lower maximal HbA1c change of 0.23 % (IQR: 0.18–0.30

Table 2
Prevalence of micro- and macrovascular disease at study enrolment in patients with newly diagnosed type 2 diabetes with and without pathogenic *GCK* variants.

	With *GCK* variant N = 17		Without *GCK* variant N = 2807		
	N patients with prevalent event	Proportion (95 % CI)	N patients with prevalent event	Proportion (95 % CI)	Crude prevalence ratio, with vs without *GCK* variant (95 % CI)
No micro- and macrovascular disease	14	82.4 (59.0; 93.8)	2259	80.5 (79.0; 81.9)	1.02 (0.82; 1.28)
Diabetes with any microvascular disease	0	0.0 (0.0; 18.4)	80	2.9 (2.3; 3.5)	0.00 (.;.)
Diabetes with any kidney disease	0	0.0 (0.0; 18.4)	29	1.0 (0.7; 1.5)	0.00 (.;.)
Diabetes with any eye disease	0	0.0 (0.0; 18.4)	31	1.1 (0.8; 1.6)	0.00 (.;.)
Diabetes with any neurologic disease	0	0.0 (0.0; 18.4)	21	0.7 (0.5; 1.1)	0.00 (.;.)
Diabetes with any macrovascular disease	3	17.6 (6.2; 41.0)	497	17.7 (16.3; 19.2)	1.00 (0.36; 2.79)
Ischemic heart disease diagnosis	2	11.8 (3.3; 34.3)	327	11.6 (10.5; 12.9)	1.01 (0.27; 3.73)
Atherosclerotic cerebrovascular disease	1	5.9 (1.0; 27.0)	143	5.1 (4.3; 6.0)	1.15 (0.17; 7.78)
Atherosclerotic peripheral vascular disease	0	0.0 (0.0; 18.4)	77	2.7 (2.2; 3.4)	0.00 (.;.)

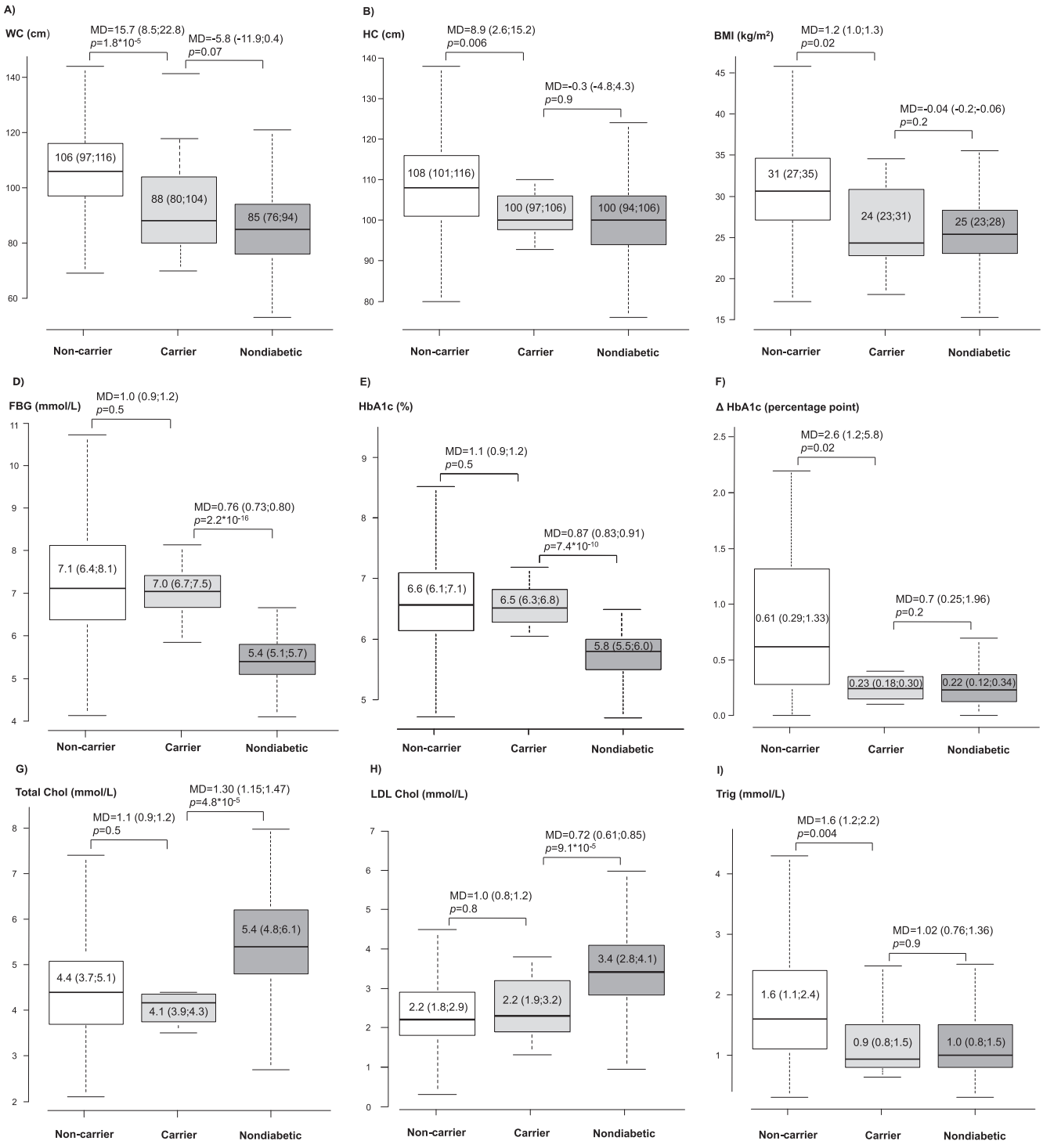

Fig. 1. Phenotypic characteristics of patients with newly diagnosed type 2 diabetes carrying *GCK* variants versus newly diagnosed type 2 diabetes patients and nondiabetic individuals not carrying *GCK* variants. Legend: Horizontal line depicts the median; the box depicts the interquartile range (IQR), and the vertical dashed line depicts the whiskers (Q1-1.5*IQR and Q3 + 1.5*IQR). MD = mean difference including 95 % CI; P = *t*-test for difference in mean. Abbreviations: WC = Waist circumference; HC = Hip circumference; FBG = Fasting blood glucose; Δ HbA1c = Maximal HbA1c change; Chol = Cholesterol; Trig = Triglyceride.

%) was found among carriers compared to a change of 0.61 % (IQR: 0.29–1.33 %) in non-carriers with diabetes resulting in a mean difference of 2.6 (95 % CI: 1.2–5.8, p = 0.02). The HbA1c change in carriers was comparable to the change found in nondiabetic individuals (0.22 % (IQR: 0.12–0.34 %)) resulting in a mean difference of 0.7 (0.25–1.96), p = 0.5 (Fig. 1 F and Supplementary Table 3).

Lipid composition was also compared between carriers and non-carriers. Total cholesterol and LDL-cholesterol among carriers [total cholesterol: 4.1 mmol/l (IQR:3.9–4.3); LDL-cholesterol: 2.2 mmol/l (IQR: 1.9–3.2 mmol/l)] and non-carriers with diabetes [total cholesterol: 4.4 mmol/l (IQR: 3.7–5.1 mmol/l); LDL-cholesterol: 2.2 mmol/l (IQR: 1.8–2.9 mmol/l)] were comparable but were significantly lower than levels observed in nondiabetic individuals [total cholesterol: 5.4 mmol/l (IQR: 4.8–6.1 mmol/l); LDL-cholesterol: 3.4 mmol/l (IQR: 2.8–4.1)] (Fig. 1 G-H and Supplementary Table 3). Levels of triglycerides in carriers [0.9 mmol/l (IQR: 0.8–1.5 mmol/l)] were lower than the levels in

Table 3
Incidence rate and incidence rate ratio of complications up to 10 years after study inclusion in patients with newly diagnosed type 2 diabetes with and without pathogenic *GCK* variants.

Complications	Number of cases with complication		Total person-years at risk		Incidence rate (95 % CI) per 100 years		IRR with vs without *GCK* variant (95 % CI)
	With *GCK* variant N = 17	Without *GCK* variant N = 2807	With *GCK* variant	Without *GCK* variant	With *GCK* variant	Without *GCK* variant	
Diabetes with any microvascular complication	3	544	103	17,252	2.92 (0.60, 8.55)	3.15 (2.89, 3.43)	0.93 (0.30, 2.88)
Diabetes with any kidney complication	1	186	113	19,772	0.89 (0.02, 4.93)	0.94 (0.81, 1.09)	0.94 (0.13, 6.72)
Diabetes with any eye complication	2	304	106	18,665	1.88 (0.23, 6.79)	1.63 (1.45, 1.82)	1.15 (0.29, 4.64)
Diabetic retinopathy	2	252	106	19,174	1.88 (0.23, 6.79)	1.31 (1.16, 1.49)	1.43 (0.36, 5.75)
Severe diabetic retinopathy	0	57	117	20,316	0.00 (0.00, 3.16)	0.28 (0.21, 0.36)	0.00 (0.00, NA)
Blindness and severe visual impairment	0	1	117	20,720	0.00 (0.00, 3.16)	0.00 (0.00, 0.03)	0.00 (0.00, NA)
Diabetes with any neurologic complication	0	130	117	19,959	0.00 (0.00, 3.16)	0.65 (0.54, 0.77)	0.00 (0.00, NA)
Expanded major atherosclerotic cardiovascular events	1	392	111	19,020	0.90 (0.02, 5.01)	2.06 (1.86, 2.28)	0.44 (0.06, 3.11)
Heart failure	0	114	117	20,320	0.00 (0.00, 3.16)	0.56 (0.46, 0.67)	0.00 (0.00, NA)
Coronary revascularization	0	189	117	19,856	0.00 (0.00, 3.16)	0.95 (0.82, 1.10)	0.00 (0.00, NA)
Unstable angina pectoris	0	50	117	20,483	0.00 (0.00, 3.16)	0.24 (0.18, 0.32)	0.00 (0.00, NA)
Myocardial infarction	0	115	117	20,206	0.00 (0.00, 3.16)	0.57 (0.47, 0.68)	0.00 (0.00, NA)
Stroke	1	112	111	20,290	0.90 (0.02, 5.01)	0.55 (0.45, 0.66)	1.63 (0.23, 11.67)
All cause death	2	221	117	20,730	1.71 (0.21, 6.19)	1.07 (0.93, 1.22)	1.61 (0.40, 6.47)

Abbreviation: IRR = incidence rate ratio.

non-carriers with diabetes [1.6 mmol/l (IQR: 1.1–2.4 mmol/l)] and were similar to the levels observed in nondiabetic individuals [1.0 mmol/l (IQR: 0.8–1.5 mmol/l)] (Fig. 1 I and Supplementary Table 3).

The number of patients, who reported parental diabetes, was significantly higher among carriers (65 %) than among non-carriers (34 %), p = 0.007 (Supplementary Table 3).

3.5. Micro- and macrovascular disease

The prevalence of micro- and macrovascular disease reported up to 10 years prior to diagnosis of diabetes was examined in patients with newly diagnosed type 2 diabetes (Table 2). Among carriers of pathogenic *GCK* variants, 82 % had not experienced any disease compared to 81 % of non-carriers. None of the carriers had experienced any microvascular diseases compared to 3 % among non-carriers. Macrovascular disease in the form of ischemic heart disease and atherosclerotic cerebrovascular disease at baseline was observed in three carriers (Table 2), but was not significantly different from macrovascular disease at baseline in non-carriers (prevalence ratio 1.00 (95 % CI 0.36–2.79) (Table 2).

Incident microvascular complications during follow-up were recorded for three patients with pathogenic *GCK* variants [incidence rate 2.92 (0.60, 8.55) per 100 years] of which two were non-severe eye complications (diabetic retinopathy) and one was a renal complication (uremia). In addition, one carrier suffered a stroke and two carriers died during follow-up (Table 3). There were no clear differences in the incidence rate of complications during follow-up when comparing carriers and non-carriers (Table 3).

3.6. Treatment

Among patients carrying a pathogenic variant in *GCK*, all except two patients were treated with metformin. Three patients received insulin in addition to metformin and two patients received another antidiabetic drug (but not insulin), in addition to metformin. Two patients did not receive any glucose-lowering treatment. Prescription patterns were also investigated among diabetes patients without pathogenic variants in *GCK* and no significant differences were observed (Table 4).

Table 4
Prescription pattern in patients with newly diagnosed type 2 diabetes with and without pathogenic *GCK*variants .

Patients with any prescription of:	*GCK* carriers		Non-carriers		
	N = 17		N = 2807		
	n (%)	95 % CI	n (%)	95 % CI	*p*-value
Any glucose-lowering drug (antidiabetics)	15 (88.2)	(65.7; 96.7)	2610 (93.0)	(92.0; 93.9)	0.4
Metformin	15 (88.2)	(65.7; 96.7)	2567 (91.4)	(90.4; 92.4)	0.6
Metformin monotherapy only	10 (58.8)	(36.0; 78.4)	1250 (44.5)	(42.7; 46.4)	0.2
Monotherapy other than metformin, or combination therapy with or without metformin (all excluding insulin)	2 (11.8)	(3.3; 34.3)	867 (30.9)	(29.2; 32.6)	0.09
Insulin-based therapy	3 (17.6)	(6.2; 41.0)	493 (17.6)	(16.2; 19.0)	1.0

4. Discussion

Non-synonymous pathogenic variants or VUS in *GCK* were 14-fold more prevalent among patients with newly diagnosed type 2 diabetes compared to nondiabetic individuals, and variants classified as pathogenic were found exclusively in patients with type 2 diabetes with a prevalence of 0.6 %. Carriers with diabetes had a leaner body composition (waist, hip, and BMI) and lower levels of triglycerides, consistent with the levels found in nondiabetic individuals. Levels of C-peptide were significantly lower among patients with pathogenic *GCK* variants compared to patients without . Repeated HbA1c measures were more stable among those with newly diagnosed type 2 diabetes patients who carried pathogenic *GCK* variants and nearly-two thirds reported parental diabetes compared to only a third of the patients with diabetes without pathogenic *GCK* variants.

The pathogenicity of variants were classified according to ACMG guidelines. Variant functionality was furthermore examined in light of a functional assay (MAVE score) and evolutionary conservation (GEMME score). While other commonly used *in silico* methods are partly trained to predict pathogenicity of variants, and are also used to assess pathogenicity, there may be some circularity and lack of independence between training and testing data in these models [31]. Both the (experimental) MAVE and (computational) GEMME scores used here each rely on a single source of data and are completely independent of any clinical annotations of variants. Thus, both are reliable in their ability to predict clinical annotations of unseen variants as well as for previous annotations, and to be independent of each other.

Functional analysis (MAVE data) supported loss-of function for seven out of eight ACMG pathogenic variants with the exception being the p.Gly318Arg variant with a value (1.3) slightly higher than wild-type-like synonymous variants (score ~ 1), suggesting that it might be mildly activating, although the score is associated with a standard error of 0.4. In our cohort, the carrier of the p.Gly318Arg variant was a patient diagnosed with diabetes and the variant has previously been found in MODY families [29]. This indicates that the pathogenicity of p.Gly318Arg is related to aspects of GCK functionality not captured by the yeast-based functional assay, e.g. cellular abundance. The GEMME score accurately predicted a deleterious effect from an evolutionary point of view in all ten missense mutations ACMG classified as pathogenic.

The ACMG classification was inadequate to assign pathogenicity to nine of the 24 identified variants. Three of these VUS appeared to be loss-of-function based on the MAVE score: p.Gly246Ala, p.His317Pro and the p.Val455Glu, which for the p.Val455Glu variant was supported by a GEMME score also indicating loss-of-function. p.Gly246Ala and p.His317Pro were both found in patients with diabetes, however, the p.Val455Glu was found in a glucose tolerant individual with a fasting plasma glucose of 5.7 and an Hba1c of 5.8 %. Thus, the p.Val455Glu does not lead to severe loss-of protein function.

Therefore, it appears that no classification method can stand alone. The ACMG classification has the challenge of the large number of VUS, the MAVE score categorised a pathogenic variant as non-functional and a benign variant as functional which was also seen for the GEMME score. Thus, considering the MAVE score and the GEMME score combined with the ACMG classification and the phenotypic information on the carrier, may facilitate a more accurate pathogenicity classification in a larger number of patients.

The 0.6 % prevalence of pathogenic variants among patients with type 2 diabetes found in this study accords well with two previous studies that investigated the prevalence of *GCK* variants in patients with type 2 diabetes. Bansal and colleagues [12] found a prevalence of *GCK* missense variants of 0.5 %, whereas Bonnefond and colleagues [13] found that 0.6 % of patients with type 2 diabetes carried pathogenic or likely pathogenic *GCK* variants. However, these studies found a higher prevalence of carriers among nondiabetic individual (0.035 % and 0.085 %, respectively), where we found no carriers of pathogenic or likely pathogenic variants in nondiabetic individuals. This may reflect that absence of diabetes was classified according to the OGTT. Thus, asymptomatic individuals with hyperglycaemia above diabetic levels were a priori excluded from the analysis of nondiabetic individuals in our study. This finding solidifies that the penetrance of pathogenic *GCK* mutations also is complete among patients with type 2 diabetes. Bonnefond and colleagues found a leaner phenotype and a lower age of diagnosis among ten patients with type 2 diabetes who carried functional *GCK* variants [13]. In the present study, we also observed a leaner phenotype, but only a slightly and non-significantly lower (~5 years) age at diagnosis. Unlike Bonnefond *et al*, we observed a significantly higher number of patients with known parental diabetes among *GCK* carriers compared to patients without *GCK* variants (65 % vs 34 %).

The phenotypic presentation of patients with type 2 diabetes carrying pathogenic *GCK* variants found in this study resembles that of MODY patients, *i.e.* a non-obesity driven diabetes with HbA1c levels between 5.6 % and 7.6 % (38–60 mmol/mol) and fasting glucose levels between 5.5 mmol/l and 8.0 mmol/l [9,33]. Glucose-lowering treatment is not efficient for the majority of *GCK*-MODY patients [33]. Nevertheless, most patients in the present study with *GCK* variants were treated for their hyperglycaemia including three individuals who received insulin in addition to metformin. Overall, the treatment regimen was similar in patients with type 2 diabetes with and without *GCK* variants, consistent with previous findings reported by Bonnefond *et al.* [13]. The modest fluctuations in HbA1c among carriers of *GCK* variants with diabetes despite treatment, is consistent with the notion that these patients are less responsive to glucose-lowering treatment.

The total number of carriers is low in the present study as the study investigates rare variants. In addition, the number of events were small. However, the prevalence and incidence of severe diabetes complications found in the *GCK* variant carriers with newly diagnosed type 2 diabetes appear similar to those in patients with *GCK*-MODY, where simplex retinopathy is observed but no carriers of *GCK* variants developed severe eye complications.

Diabetes may have more than one underlying cause. However, based on the current study, we suggest that the *GCK* variants are causal in most of the patients who carry them, especially in patients carrying pathogenic *GCK* variants. For these patients, treatment recommendations and prognosis should correspond to those of *GCK*-MODY patients. For the 17 patients who carried a pathogenic variant in the present study, this would mean discontinuation of pharmacological treatment and less frequent outpatient visits due to a low risk of complications. Furthermore, there are direct implications for the carrier's family as genetic screening of family members with diabetes should be initiated [9,34].

Five patients carried variants with undetermined pathogenicity (VUS). As sequencing is becoming an increasingly routine part of clinical

practice, our knowledge of the effect of individual variants will evolve. Thus, the certainty with which we can identify patients with causal *GCK* variations will improve. However, at this point, treatment termination in patients carrying these uncertain variants must be closely monitored, and the clinician must be ready to continue treatment, if glucose values increase beyond those known in *GCK*-MODY.

Diagnosing diabetes patients with variants in *GCK* is economically and clinically relevant. In Denmark, the healthcare cost of diabetes is ~ 1,800 USD per person-year for patients with minor complications [35]. The total Danish type 2 diabetes population encompasses approximately 270,000 patients [36]. Extrapolating the prevalence of pathogenic *GCK* variations from the present study population suggests that close to 1,600 (0.6 %x270,000) Danish patients have diabetes due to *GCK* variants. Correct diagnosis of these patients with subsequent discontinuation of treatment and lowered intensity of clinical follow-up has the potential for a reduction of ~ 4.0 million USD/year in the cost of diabetes care in Denmark (1,800 USD per 1,600 patients). The price of genetic diagnostics is currently 300 USD per sample in Denmark. When whole genome sequencing in the future will become generally available in clinical care, information on the glucokinase gene can be extracted without additional cost.

As a group, patients with newly diagnosed type 2 diabetes carrying *GCK* variants slightly differ phenotypically from patients without *GCK* variants. However, at the individual level, there are no clear phenotypic traits that could guide referral for genetic testing. We therefore suggest that genetic evaluation should be considered in non-obese patients with type 2 diabetes having an HbA1c below 7.6 % (60 mmol/mol) and fasting plasma glucose below 8 mmol/l.

4.1. Conclusion

Close to 1 % of patients with newly diagnosed type 2 diabetes are carriers of functional non-synonymous variants in *GCK*. Carriers resemble patients with *GCK*-MODY. Considering the benefit for the patient, their family members, and society, sequencing should be considered in all patients with non-obese type 2 diabetes and stable marginally increased glycaemia.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions

AL, JSN, HTS, IV, HBN, AV, JR, OP and TH were responsible for conception. APG, CTH, LE, and TH were responsible for design of the study. AL, JSN, HTS, IV, HBN, AV and JR were responsible for sample collection. APG, CTH, LE, ACBT, MH, NG, LBC, RWT and TH took part in the analyses. APG, OP, NG and TH were involved in funding acquisition. APG and TH drafted the article. APG, CTH, LE, ACBT, MH, NG, LBC, RWT, JSN, HTS, AV, OP and TH critically revised the manuscript and contributed to the discussion. The final version of the paper was read and approved by all authors.

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Appendix A. Supplementary data

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