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C679X loss-of-function *PCSK9* variant lowers fasting glucose levels in a black South African population: A longitudinal study

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ABSTRACT

Aims: To determine the longitudinal association of the loss-of-function (LOF) *PCSK9* variants (C679X and A443T), proxies of PCSK9 inhibitor drugs, with LDL-C, fasting glucose and glycated hemoglobin.

Methods: We conducted a five year, longitudinal study, nested within the Prospective Urban and Rural Epidemiology study, among 737 apparently healthy, male and female black South Africans of the North West province. Genotyping of the C679X and A443T *PCSK9* variants was achieved using Taqman assays from Applied Biosystems. Generalized estimating equations were used to determine longitudinal association of the A443T and C679X *PCSK9* variants with LDL–C, fasting glucose and glycated hemoglobin.

Results: C679X and A443T variant carriers were associated with significant reductions in LDL-C of −0.98(−1.29, −0.67) mmol/L; $p < 0.001$) and −0.39(−0.57, −0.20) mmol/L; $p < 0.001$) respectively, compared to the non-carriers. Only C679X variant was independently associated with reductions in fasting glucose of −0.37 (−0.61, −0.13) mmol/L; $p = 0.002$) compared to non-carriers. However, the association of the selected variants with glycated hemoglobin were not significant. C679X and A443T carriers were associated with −0.07 (−0.23, 0.09) %; $p = 0.400$), 0.05 (−0.13, 0.22) %; $p = 0.599$) of glycated haemoglobin respectively.

Conclusion: Our results indicated that carriers of A443T and C679X variants exhibit sustained low LDL-C levels over 5 years and have varied effects on T2D biomarkers compared to non-carriers.

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1. Introduction

Elevated levels of low density lipoprotein cholesterol (LDL-C) are a major risk factor for the development of cardiovascular diseases (CVDs) [1]. The PCSK9 inhibitor drug lowers LDL-C by 60% and has been dubbed, the greatest advancement in lipid therapy in the past 30 years [2–4]. However, there are now concerns that these drugs might be associated with increased T2D risk [5]. Although a recent meta-analysis of clinical trials for the PCSK9 inhibitor drugs indicated no effect of T2D risk, in which 23% of the patients were diabetic, there is concern that the T2D risk might be more pronounced in the future after prolonged treatment, as was noticed for statin drugs [6–9]. However, it is anticipated that some answers to the long term effects of these PCSK9 inhibition drugs might be found by assessing T2D risk among carriers of the *PCSK9* variants which are proxies for these drugs [8].

The development of PCSK9 inhibitor drugs was motivated by the discovery of LOF *PCSK9* variants that are associated with low LDL-C levels from childhood to adulthood in African Americans and Caucasians [10–12]. Notably, strong evidence favouring this approach, came from African Americans who were compound heterozygote carriers of (Y142X and ΔR97) *PCSK9* variants and had no circulating PCSK9 [10]. However most of these earlier studies on *PCSK9* variants, focused on LDL-C levels and other lipid traits, ignoring the association of these variants with markers of T2D risk. Recently, studies among Caucasians have evaluated the association of *PCSK9* variants with T2D risk and yielded conflicting results. A cross-sectional study indicated that the *PCSK9* variant R46L did not affect T2D risk [13]. By contrast, a recent meta-analysis and a Mendelian randomisation study revealed increased T2D risk associated with selected LOF *PCSK9* variants that comprised of R46L [5,11,13].

Assessments of the association of LOF *PCSK9* variants with T2D risk have been explored only among Caucasians with regards to T2D risk [8]. Therefore more studies of the association of LDL-C lowering PCSK9 variants (A443T, C679X, Y142X) common in people of African ancestry with T2D risk are required. This study set out to determine the longitudinal association of A443T and C679X *PCSK9* variants with LDL-C, fasting glucose, glycated hemoglobin over a 5 year period in a black South African population.

2. Materials and methods

2.1. Study sample

The longitudinal study was nested in the South African arm of the Prospective Urban and Rural Epidemiological (PURE) study. The PURE study is a large-scale epidemiological study, which recruited approximately 140 000 participants from about 600 communities in 17 low, middle, and high income countries around the world [14]. The South African arm of the PURE study was initiated in 2005, and it has five-year follow-up intervals up to 2015. The participant data collection comprised of medical history, lifestyle behaviour (physical activity and dietary profile), blood collection (for both genetic and biochemical analyses), an electrocardiogram, and anthropometric measures. Detailed information for four specific environmental factors of importance, which consists of the level of urbanisation, nutrition and associated food policy, psychosocial/socioeconomic factors and tobacco usage is also determined in the PURE study [14].

Apparently healthy male and female volunteers who were older than 35 years, not using any chronic medication and or who had not been diagnosed with non-communicable diseases were enrolled into the PURE study in 2005. These volunteers were recruited into the South African arm of the PURE study from two rural sites and two urban sites in the North West province of South Africa. The volunteers self-identified themselves as Tswana speaking and this was regarded as their ethnicity.

For this study, 737 male and female participants were enrolled on the basis that they had biochemical data for LDL-C, fasting glucose and glycated hemoglobin in 2005 and 2010. The participants gave informed consent to participate in the study and they were allowed to exit the study when they deemed it necessary. This study was conducted in harmony with the principles of the Declaration of Helsinki and ethical approval was granted by the Ethics Committee of the North-WestUniversity (Ethics number: NWU-00016-10-A1).

2.2. Anthropometry

The anthropometric measurements were performed by the PURE research team members using standard procedures [14]. The height was assessed using a stadiometer (IP 1465, Invicta, and London, UK) and weight measurements conducted using portable electronic scales (Precision Health Scale, A&D Tokyo, Japan). Three measurements were taken and the mean was recorded. The body mass index (BMI) was computed as BMI = weight (kilograms)/height^2 (metres).

2.3. DNA extraction

Fasting blood samples were collected from the consenting volunteers by trained research nurses of the PURE South African arm study team. Deoxyribonucleic acid (DNA) isolation was performed with the use of Qiagen® Flexigene™ DNA extraction kits (catalogue number 51206; QIAGEN Pty. Ltd., Australia). The DNA yield was evaluated using the NanoDrop™ spectrophotometer (ND-1000, Wilmington, DE, USA) and then normalised to a concentration of 10 ng μL^{-1} for genotyping.

2.4. Genotyping

The Taqman SNP Genotyping Assays by Applied Biosystems (Carlsbad, CA, USA) for C679X (rs28362286: C > A – Assay ID: C_63745519_10) and A443T (rs28362263: G > A – Assay ID: C_25934978_20) were used for the genotyping with the CFX96 Real-Time PCR detection system (Bio-Rad Laboratories Inc., Singapore). Sanger sequencing was done to validate the compound heterozygotes. Variant A443T is located in exon 8

and the following forward primer: ATCACCATCTTTCACCATTC and reverse primer: GGTACAGTCACCTCCATG were used during the amplification process. Variant C679X is situated in exon 12 of the *PCSK9* gene and the following forward primer: GGAGGGAGAAATGAAGTG and the reverse primer: TAGATGCCATCCAGAAAG were used for the amplification reaction. The Sanger sequencing was done by the Central Analytical facility at Stellenbosch University. The CLC workbench version 6.84 (Qiagen/CLC Bio, Canada) was used to align the DNA sequence reads and to determine the genotypes of the carriers of the compound heterozygotes of A443T and C679X.

2.5. *Biochemical analysis*

The Konelab20iTM auto analyser (Thermo Fisher Scientific Oy, Vantaa, Finland) was used to measure the high density lipoprotein cholesterol (HDL-C), total cholesterol (TC) and triglycerides (TG) from the serum of the participants. The Friedwald equation (Friedwald equation [LDL-C = TC − HDL-C − (TG)/2.17]) was used to determine the LDL-C levels of the volunteers. Fasting glucose and glycated hemoglobin were assessed using SYNCHRON® System and the Bio-Rad D-10TM machine (Bio-Rad Laboratories, Inc., Hercules, France) respectively as described elsewhere [15].

2.6. *Statistical analysis*

The Q-Q plots of the continuous variables were used to assess normality of the continuous variables. The Statistical package for Social Scientist (SPSS) version 24 and Stata 13 were used for all the statistical analysis. For the descriptive statistics, the continuous variables were compared among the *PCSK9* variant carriers and the non-carriers using ANOVA. Categorical variables (gender, sex and urbanisation) were compared among the (A443T, C679X, CH) *PCSK9* variant carriers groups and the non-carriers using chi-square tests. The adherence to the assumptions of Hardy Weinberg equilibrium (HWE) of the *PCSK9* variants was assessed using chi-square tests ($p < 0.05$). The missingness of the data was evaluated using the Little MCAR test in SPSS. The compound heterozygotes were not included in the analysis, since there were only 3 and deemed less representative. The PCSK9 composite variable was recoded to indicate carriers of and non-carriers of A443T and C679X variants as indicated in Tables 2 and 3. Generalised estimating equations (GEE) models were used to assess the longitudinal association of *PCSK9* variant carriers with LDL-C levels, fasting glucose and glycated hemoglobin after the missing data was noted to be missing completely at random (MCAR). The crude models of the PCSK9 variants with the dependent variables were depicted as model 1. Model 2 comprised of the PCSK9 variants with the fixed factors and covariates as illustrated in Tables 2 and 3. The continuous variables were treated as covariates and the categorical variables as fixed factors in these models. Participants on diabetes and cholesterol medication were removed from the analysis. The mediation and or confounding of BMI on the effect of *PCSK9* variants on the metabolic markers was evaluated by: (i) assessing the longitudinal association of the *PCSK9* variants with BMI (ii) including BMI independently and together with *PCSK9* variants in the models for the longitudinal association of the selected biomarkers. (iii) The interaction of BMI and the PCSK9 variants were assessed in the longitudinal analysis models of the selected biomarkers.

3. Results

3.1. *Descriptive results*

The minor allele frequencies for A443T and C679X variant alleles were 4.45% and 2.35%, respectively. All these variants were in Hardy Weinberg equilibrium and the genotyping rate was 98.5% after quality control. Three compound heterozygotes for the A443T and C679X variants were identified and validated through Sanger sequencing. There was a significant difference in total cholesterol (TC) levels, LDL-C levels and urban/rural residence status of the carriers of the *PCSK9* variant carriers and the non-carriers as indicated in Table 1. The mean unadjusted differences in LDL-C levels over the 5 years between the *PCSK9* variant carriers and the non-carriers were 57%, 30%, 16% ($p < 0.001$) for compound heterozygotes, C679X and A443T carriers respectively (Fig. 1). There were differ-

Table 1 – Descriptive statistics of the carriers and non-carriers of the A443T and C679X variants of *PCSK9*.

Variable	Non-carrier	Carriers			P value
		A443T	C679X	CH	
N (%)	644 (87.38)	60 (8.14)	30 (4.07)	3 (0.41)	
Age 2005 (Years)	50.15 ± 10.25	48.72 ± 9.70	50.87 ± 9.11	51.33 ± 8.74	0.721
Age 2010 (Years)	55.36 ± 10.47	52.51 ± 9.44	55.19 ± 9.15	56.00 ± 9.17	0.673
Male Sex N (%)	238 (37)	25 (41.7)	8 (26.7)	1 (33.3)	0.582
Urban N (%)	369 (57.30)	47 (80)	23 (73.30)	3 (100)	0.001
TC 2005 (mmol/l)	5.17 ± 1.30	4.65 ± 1.35	4.29 ± 1.17	4.49 ± 0.99	<0.001
TC 2010 (mmol/l)	4.97 ± 1.17	4.52 ± 0.97	4.06 ± 1.11	3.10 ± 1.43	<0.001
HDL-C 2005 (mmol/l)	1.50 ± 0.57	1.45 ± 0.62	1.49 ± 0.76	1.50 ± 0.88	0.948
HDL –C 2010 (mmol/l)	1.42 ± 0.56	1.42 ± 0.61	1.54 ± 0.77	1.36 ± 0.42	0.700
BMI 2005 (kg m^{-2})	24.87 ± 6.78	24.91 ± 7.80	24.38 ± 5.65	22.19 ± 8.15	0.895
BMI 2010 (kg m^{-2})	25.38 ± 7.16	25.45 ± 7.70	24.63 ± 6.78	22.16 ± 7.13	0.822
Diabetes Medications 2010 (N (%))	33 (5.2)	2 (3.4)	2 (6.7)	0	0.711

TC = total cholesterol; HDL-C = high density lipoprotein cholesterol; BMI = body mass index. CH = compound heterozygotes. Mean ± standard deviation is reported for the continuous variables.

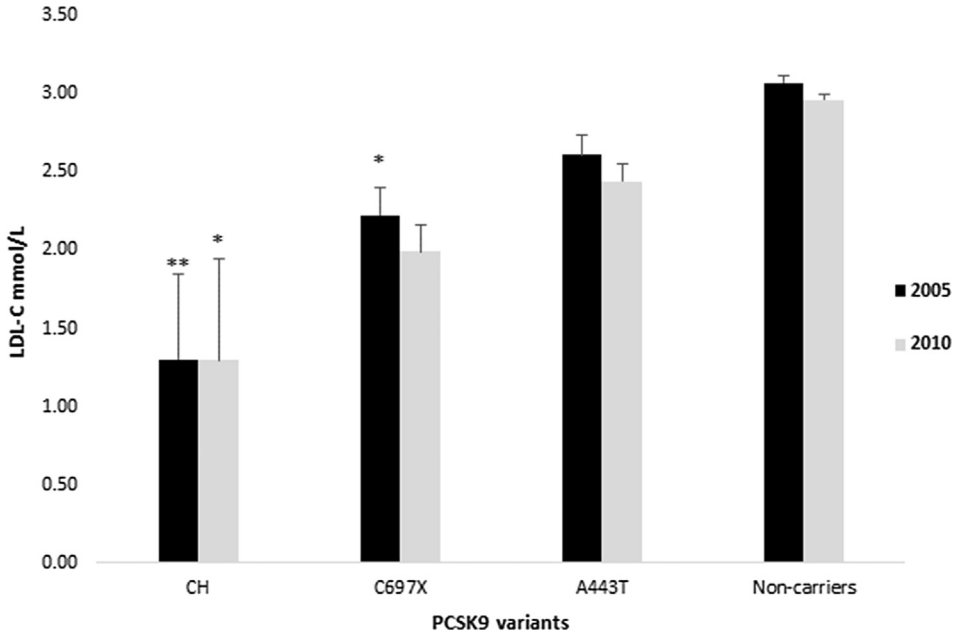

Fig. 1 – Unadjusted LDL-C levels of the PCSK9 variant carriers and non-carriers, overall p < 0.001 for both years. The values are presented as mean ± SEM. ** = p < 0.01; * = p < 0.05.

ences that were noted in the unadjusted means of fasting glucose for the carriers and non-carriers of the selected PCSK9 variants as illustrated in Fig. 2. However, these were not significant.

3.2. *Longitudinal associations of PCSK9 variants with LDL-C, fasting glucose and glycated hemoglobin*

The *PCSK9* variants assessed in this study were all significantly associated with lower LDL-C levels as compared to the non-carriers, with the lowest effect of −0.91 mmol/L among the C679X carriers (Table 2). Significant associations of 0.37 mmol/L lower fasting glucose levels compared to the non-carriers were seen for C679X carriers (Table 3). However, no significant association of the *PCSK9* variant carriers with glycated hemoglobin were noted. The longitudinal associations of the selected PCSK9 variants with the metabolic biomarkers were not mediated but independent from BMI as indicated in the Supplementary Tables 1–3.

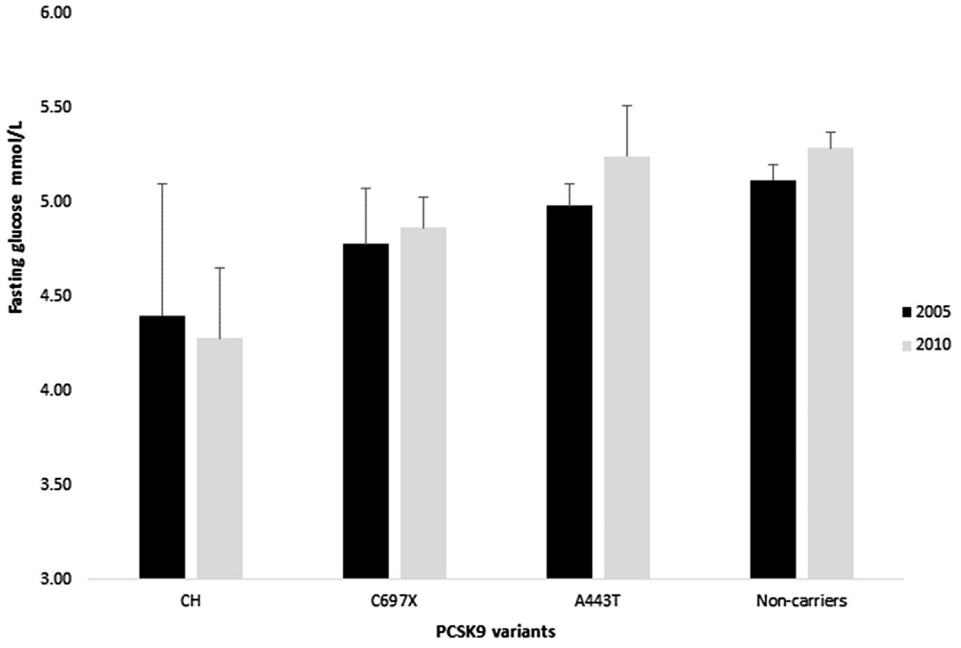

Fig. 2 – Unadjusted Fasting glucose levels of the *PCSK9* variant carriers and non-carriers. The values are presented as mean ± SEM.

Table 2 – Adjusted longitudinal association of *PCSK9* variants with LDL-C.

Variable	Model 1		Model 2	
	β (95% CI)	P value	β (95% CI)	P value
Non Carrier	Reference		Reference	
A443T	−0.43 (−0.63, −0.23)	<0.001	−0.39 (−0.57, −0.20)	<0.001
C679X	−0.91 (−1.24, −0.58)	<0.001	−0.98 (−1.29, −0.67)	<0.001
Female (vs Male)			−0.23 (−0.38, −0.08)	0.002
Age			0.01 (0.01, 0.02)	<0.001
Time 2010 (vs 2005)			0.17 (0.09, 0.25)	<0.001

4. Discussion

We report for the first time the longitudinal association of C679X and A443T variants with fasting glucose and glycated hemoglobin. Notably, the compound heterozygotes (for C679X and A443T) and C679X carriers had significantly lower fasting glucose levels of 0.94 mmol/L and 0.37 mmol/L per risk allele, respectively compared to the non-carriers. However, there were no significant associations of the *PCSK9* variant carriers with glycated hemoglobin. In the current investigation, we replicated the association of the A443T and C679X *PCSK9* variants with low LDL-C levels in a black South African population [10]. The compound heterozygote carriers of these variants were significantly associated with 1.61 mmol/L lower LDL-C levels per risk allele compared to the non-carriers.

The association of A443T and C679X *PCSK9* variants with low LDL-C levels has been described in detail in a number of cross-sectional and longitudinal studies of both young and old participants [12,16–20]. Our findings are in line with regards to the body of knowledge in this domain and we will focus on elaborating the novel aspects of our study. We identified three compound heterozygotes of the A443T and C679X *PCSK9* variants for the first time. These carriers had mean LDL-C levels of 1.30 mmol/L which were 57% lower than those of non-carriers over a 5-year period. However, the mean LDL-C levels of the compound heterozygotes we identified were slightly higher than those determined in an African American individual for the *PCSK9* Y142X and ΔR97 variants, which were noted to be 0.78 mmol/L [21]. However, the discrepancy in the low LDL-C levels associated with the *PCSK9* Y142X and ΔR97 compound heterozygote and the A443T and C679X *PCSK9* compound heterozygotes is not surprising as these mutations have different effects on the synthesis, processing and action of the PCSK9 molecule. The Y142X and the ΔR97 prevents the secretion of the PCSK9 molecule through disrupting its synthesis and autocatalytic cleavage respectively, making the PCSK9 not detectable in the blood stream [21]. On the hand, the C679X variant prevents the secretion of PCSK9 by disrupting its normal folding, while the A443T variant makes the mature PCSK9 molecule more susceptible to furin cleavage thereby making it lose a larger segment of its catalytic chain and thus become inactive [21,22]. However, carriers of these variants have detectable PCSK9 levels. Thus their combined effect can be expected to be lower than that of the Y142X and ΔR97 compound heterozygote carriers that have undetectable PCSK9 levels.

Our findings of reduced fasting glucose levels associated with the compound heterozygotes (A443T and C679X) and the C679X variant are different from what has been reported for R46L in Caucasian populations [5,8,11]. There was no significant association between BMI and the selected *PCSK9* variants (Supplemental Table 1). When BMI was included in the models for fasting glucose, the effects sizes of the *PCSK9* variants did not change much and their association with fasting glucose remained significant and the interaction of the variants with BMI was not significant (Supplemental Table 2). Therefore the association of the selected *PCSK9* variants with fasting glucose is independent of BMI. However, the heterozygous carriers of the A443T had no significant effects on the evaluated T2D biomarkers, but modest increases of glycated hemoglobin were noted for them. The non-significant effects for glycated hemoglobin noted in this study were previously reported by Schmidt et al. 2017 [5]. Although the absence of a mechanism behind the association of LOF *PCSK9* with T2D risk limits the interpretation of these findings, the correlations of PCSK9 with fasting glucose and glycated hemoglobin may help clarify the discrepancies in the relationships of these variants with markers of disglycemia [8]. The R46L and A443T heterozygotes were noted not to affect PCSK9 levels, while the C679X lower levels of PCSK9 [21,23]. It has been reported that low PCSK9 levels are protective of T2D and high PCSK9 levels are associated with incident T2D [23,24]. A positive correlation between PCSK9 levels and fasting glucose has been reported [25]. Therefore, the C679X variants which lower PCSK9 levels were associated with reduced fasting glucose levels.

Our study was limited by the few compound heterozygotes we identified, due to their scarcity. Nonetheless, the compound heterozygotes were better proxies for the PCSK9 inhibitors due to their 57% LDL- C levels lowering effect. However, our results are preliminary and need to be validated in larger studies among people of Africa ancestry. Variants such as C679X, Y142X and the ΔR97 that have large effects should be prioritised in the genetic risks scores evaluating the association of *PCSK9* variants and T2D risk in these studies [10,21]. However, the absence of the knowledge of the mechanism of the *PCSK9* variants with T2D limits the application of these findings to PCSK9 inhibitor drugs. The varied effects of the LOF *PCSK9* variants on T2D biomarkers further complicates this phenomenon [8]. Therefore, future longitudinal studies of the patients using the PCSK9 drugs will be required to validate the associations of these drugs with T2D risk.

In summary, this pioneering longitudinal study has documented the association of A443T and C697X *PCSK9* variants with LDL-C, fasting glucose and glycated hemoglobin. The

Table 3 – Adjusted longitudinal association of PCSK9 variants with fasting glucose.

Variable	Fasting glucose				Glycated hemoglobin			
	Model 1		Model 2		Model 1		Model 2	
	β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value	β (95% CI)	P value
Non carrier	Reference		Reference		Reference		Reference	
A443T	−0.03 (−0.32, 0.27)	0.862	0.01 (−0.29, 0.30)	0.966	0.01 (−0.16, 0.19)	0.868	0.05 (−0.13, 0.22)	0.599
C679X	−0.32 (−0.56, −0.08)	0.007	−0.37 (−0.61, −0.13)	0.002	−0.02 (−0.19, 0.15)	0.812	−0.07 (−0.23, 0.09)	0.400
Female (vs Male)			−0.28 (−0.42, −0.14)	<0.001			−0.28 (−0.37, −0.19)	<0.001
Age			0.01 (0.00, 0.02)	0.040			0.01 (0.00, 0.02)	0.001
Time 2010 (vs 2005)			0.14 (0.02, 0.26)	0.021			0.35 (0.29, 0.42)	<0.001

C679X variant was associated with lower fasting glucose levels contrary to what had been reported for variants such as R46L in Caucasians. The A443T variant was associated with modest increases in glycated hemoglobin though not significant, unlike the C679X variant. Thus the LOF PCSK9 variants that lower LDL-C have varied effects on T2D risk biomarkers which limits their use as proxies of PCSK9 inhibitor drugs in this phenomenon. However, more studies of the C679X, Y142X and the ΔR97 variants which motivated the development of the PCSK9 inhibitor drugs are required to further explore this phenomenon.

Conflict of interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.diabres.2018.09.012.

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