Visit-to-Visit Variability of Hemoglobin A₁c in People Without Diabetes and Risk of Major Adverse Cardiovascular Events and All-Cause Mortality

https://doi.org/10.2337/dc18-1396

OBJECTIVE
We aimed to study whether visit-to-visit variability of glycated hemoglobin A₁c (HbA₁c) is associated with incident major adverse cardiovascular events (MACE), all-cause mortality, and type 2 diabetes in people without diabetes.

RESEARCH DESIGN AND METHODS
We included primary care patients with no history of diabetes or cardiovascular disease and with three annual HbA₁c measurements within normal range (<6.5% [48 mmol/mol]). For each individual, we measured the HbA₁c variability as the SD of the residuals obtained from a linear regression on the three HbA₁c measurements. From the linear regression, we also obtained the estimated index HbA₁c (intercept) and the trend over time (slope). Follow-up began at the date of the third measurement. Associations between HbA₁c variability and outcome were analyzed using Cox regression, adjusted for traditional risk factors, intercept, and trend, and reported as hazard ratio per SD increase in variability (HRSD).

RESULTS
In total, 6,756 individuals were included. During a median follow-up time of 6.3 years, 996 developed MACE, 856 died, and 1,267 developed type 2 diabetes. We found a significant association between increasing HbA₁c variability and incident MACE (HRSD 1.09 [95% CI 1.03–1.15]) and all-cause mortality (HRSD 1.13 [95% CI 1.07–1.20]), whereas there were no associations with type 2 diabetes (HRSD 1.00 [95% CI 0.95–1.05]). We calculated 5-year absolute risks of MACE and all-cause mortality and found clinically relevant differences across several age, sex, and comorbidity subgroups.

CONCLUSIONS
In a primary care population free of diabetes and cardiovascular disease, high HbA₁c variability was associated with increased risks of MACE and all-cause mortality.

Type 2 diabetes is an important risk factor for major adverse cardiovascular events (MACE) and premature death from cardiovascular causes (1,2). Glycated hemoglobin A₁c (HbA₁c) is a readily available and highly reproducible biomarker that reflects the 3-month average plasma glucose concentration; it can be measured in the nonfasting state, and a value ≥6.5% (48 mmol/mol) is considered diagnostic for diabetes (3).
In addition to high HbA1c (4–8), recent observational studies have also shown that high long-term glycemic variability, measured as intrapatient variability in HbA1c over time, is associated with micro- and macrovascular complications, independently of mean HbA1c levels, both in patients with type 1 and type 2 diabetes (9–13). The clinical utility of variation in HbA1c as a marker for microvascular or macrovascular complications has yet to be established, but the findings are supported by data from in vitro and animal studies indicating that a high plasma glucose variation has a greater effect on endothelial function and oxidative stress generation compared with a sustained high glucose level (14–16). Whether glycemic variability in people without diabetes is an independent risk factor for future cardiovascular events and mortality or even type 2 diabetes is currently unknown.

Our objective was to evaluate the relationship between visit-to-visit variability in HbA1c and the risk of MACE, all-cause mortality, and incident type 2 diabetes in adults without diabetes.

**RESEARCH DESIGN AND METHODS**

**Study Population**

Most general practitioners practicing in Copenhagen, Denmark, refer their patients to one core facility (Copenhagen General Practitioner’s Laboratory [CGPL]) for a large variety of clinical tests, including blood sampling for biochemical analyses. For the current study, we identified all individuals who had at least one HbA1c measurement taken between 1 January 2003 and 31 December 2013. Individuals were only included if they, in addition to the first measurement, had two consecutive measurements, one after 1 year (±3 months) and another after 2 years (±3 months) (Supplementary Fig. 1). The date of the first measurement corresponds to the index date. The baseline date for follow-up was set at the third measurement of HbA1c. Patients were excluded if they 1) were <18 or >100 years of age at index date, 2) had any HbA1c measurement ≥6.5% (48 mmol/mol) prior to baseline, or if they prior to baseline were diagnosed with 3) any type of diabetes (or were prescribed antidiabetic medication), 4) ischemic heart disease, 5) ischemic stroke or transient ischemic attack, or 6) peripheral vascular disease. A detailed description of the HbA1c assays and quality assessment has been published previously (17).

**Data Sources**

All residents in Denmark are assigned a unique and permanent civil registration number that enables linkage of administrative registries on an individual level. The Danish National Patient Registry holds nationwide information on all hospital, outpatient clinic, and emergency room admissions since 1978. At discharge, each hospitalization or visit at an outpatient clinic or emergency room is registered with one primary diagnosis and, if applicable, one or more secondary diagnoses according to the ICD. The ICD-8 was used from 1978 to 1994 and the ICD-10 was used from 1995 and onwards. The Danish Register of Medicinal Products Statistics holds nationwide information on all dispensed prescriptions from Danish pharmacies since 1995 based on the Anatomical Therapeutic Chemical System (ATC) (18). Sex, date of birth, and death along with date of emigration and causes of death can be obtained from the Danish Central Personal Registry. Annual data on personal income were provided by Statistics Denmark (19). The cause of death, classified by ICD codes, can be obtained from the Danish Register of Causes of Death (20). In accordance with Danish law, no approval from an ethics committee is needed in a registry-based study without any active participation from study subjects. The use of deidentified registry data was approved by the Danish Data Protection Agency (record number 2007-58-0015).

**Baseline Variables and Follow-up**

We defined hypertension from discharge diagnosis or if a subject prior to baseline was treated simultaneously with at least two types of antihypertensive drugs (Supplementary Tables 1 and 2). The Charlson Comorbidity Index (CCI) was used to account for the general morbidity burden (21). The CCI takes multiple comorbidities into account, including chronic obstructive pulmonary disease, various cancer diseases, liver diseases, renal disease, and cardiovascular diseases (Supplementary Table 3). Based on the total score, we categorized patients into levels of no comorbidity (score 0) and comorbidity (score ≥1). The score has been reported to have high discrimination in predicting mortality (21,22). We separately ascertained contacts for obesity- and smoking-related disorders (23) and used these as covariates in a sensitivity analysis as described below. Smoking was defined from discharge diagnosis for smoking or chronic obstructive pulmonary disease, or pharmacological or nonpharmacological treatment for smoking cessation. We defined socioeconomic status as the individual mean annual gross income during a 5-year period prior to baseline (24). Income was categorized into tertiles. The end points of interest were incident MACE, death from all causes, and type 2 diabetes. MACE represented a composite end point of myocardial infarction, unstable angina pectoris, ischemic stroke, peripheral vascular disease, or cardiovascular death, whichever came first. The diagnoses of myocardial infarction and ischemic stroke have previously been validated with high predicted values of 93.6% and 79%, respectively (25,26). Information on cause of death was extracted from the Danish Register of Causes of Death (20). The type 2 diabetes end point was defined from discharge diagnoses of type 2 diabetes and/or treatment with oral antidiabetics, whichever came first. This algorithm has previously been shown to have a sensitivity of 96% and a positive predictive value of 89% for identifying people with type 2 diabetes (27). The algorithms and registry diagnoses used for covariates and outcomes are listed in Supplementary Tables 1 and 2.

**Statistical Analyses**

The primary exposure variable of interest was visit-to-visit HbA1c variability defined as the intrapatient variability in HbA1c levels. The variability was defined as the SD of the residuals obtained from a linear regression analysis of the three HbA1c measurements of each individual, similar to what has been done previously (28). From the linear regression analysis, we used the intercept, the slope, and the SD of the residuals to characterize the patients according to index level, trend, and variability, respectively. Index HbA1c was categorized into levels of low HbA1c (<5.3% [34 mmol/mol]), moderate HbA1c (5.3–5.7% [34–39 mmol/mol]), and high HbA1c (≥5.7% [39 mmol/mol]). We categorized the slope into decreasing (less than −0.11%/year), stable (−0.11 to
Regression analyses included main and interactions (Supplementary Fig. 3). All Cox risk factors were assessed independently, with increasing, stable, and increasing trends (HbA1c, stable trend, and all combinations). We constructed a risk chart that displays the highest absolute 5-year risks. We also did sensitivity analyses (see below).

Time zero for all time-to-event analyses was set at the date of the third HbA1c measurement (baseline). Individual follow-up ended in case of the event of interest, death, emigration, or at 31 December 2015, whichever occurred first. Multiple cause-specific Cox regression models were used to evaluate the association between HbA1c variability and the hazard rates of MACE, all-cause mortality, and type 2 diabetes. Cause-specific hazard ratios (HRs) were reported for a 1-SD increase in variability (Supplementary Fig. 2). To test the linearity assumption, we used restricted cubic splines plots to assess the functional relationship between variability and the end points (Supplementary Fig. 3). All Cox regression analyses included main and interaction effects of the index HbA1c level (low, moderate, and high) and the trend (decreasing, stable, and increasing) and were further adjusted for main effects of age-groups (<50, 50–60, 60–70, 70–80, and >80 years), sex, baseline hypertension status (yes or no), and CCI (0 and ≥1).

The 5-year risk of all-cause mortality was predicted based on the Cox regression model for the hazard rate of all-cause mortality. By combining a Cox regression model for the competing risk of noncardiovascular mortality with the Cox regression model for the hazard rate of MACE, we predicted the 5-year absolute risks of MACE (29). Absolute 5-year risks were reported with 95% confidence limits for selected risk factors defined by age-group, sex, hypertension, CCI, index HbA1c group (low, moderate, and high), and trend (decreasing, stable, and increasing). Three risk profiles were defined based on the combination of risk factor values associated with the lowest, median, and highest absolute 5-year risks. We also constructed a risk chart that displays 5-year absolute risks of MACE and all-cause mortality for a moderate index HbA1c stable trend, and all combinations of the other risk factors.

The potential effect modifications of association between variability and the outcome hazard rates by age, sex, CCI, hypertension, index HbA1c, and trend were evaluated with likelihood ratio tests comparing the main model to a model with the interaction term. A two-sided \( P < 0.05 \) was considered statistically significant.

**Sensitivity Analyses**

To test the robustness of our analyses, we conducted multiple sensitivity analyses. First, patients who increased in CCI from first HbA1c measurement to last HbA1c measurement were excluded. This was done to assess whether HbA1c variability is merely a reflection of increased morbidity. Second, to test whether HbA1c variability is a measure of generalized frailty, secondary to, for example, cancer, we excluded individuals with CCI ≥1 at baseline. This is also of importance, since, for example, severe renal diseases and compromised bone marrow function are associated with altered red blood cell production and life span, affecting the validity of HbA1c as a marker of long-term blood glucose level. Third, to assess whether the effect of HbA1c variability on the hazard rate of MACE and all-cause mortality could be confounded by weight and smoking patterns, we adjusted for separately ascertained registry diagnoses of obesity and smoking-related disorders. Fourth, to assess whether a more liberal time-related inclusion criterion might influence the results, we included individuals with three measurements, annually spaced ±4 months. Fifth, to assess whether the results are dependent on the number of measurements used in the analyses, we conducted a separate analysis, including individuals with four annually spaced measurements ±3 months. Sixth, to assess whether the results could be reproduced using another marker for glucose levels, we used random blood glucose variability (RPG) as exposure. From the CGPL database, we identified all individuals who had three or more measurements of RPG, taken in the nonfasting state and annually spaced (±3 months). Individuals with any measurement ≥11.1 mmol/L (≥200 mg/dL) were excluded. All other exclusion criteria, variability measurements, intercept, and trend were obtained using the same methods as described for the main analyses. Seventh, we also used a different index for HbA1c variability, recently published by Forbes et al. (30). Here, HbA1c variability was defined as the number of times successive measurements differed by ≥0.5% (5.5 mmol/mol), divided by the number of comparisons and then multiplied by 100. Eighth, we used different cutoffs for categorization of the slope estimate to assess whether the position of the cut-off could influence the results.

**RESULTS**

**Baseline Demographics and Clinical Characteristics**

The greater region of Copenhagen has a current population of 1.3 million citizens. At CGPL, 390,747 adults underwent HbA1c testing. Of the individuals referred for HbA1c testing, 118,476 individuals had at least three measurements of HbA1c, taken between 2003 and 2013. A total of 6,756 individuals were eligible for inclusion. Supplementary Fig. 4 summarizes the number of included and excluded study subjects. Baseline clinical characteristics are summarized in Table 1.

**HbA1c Variability and Risk of Incident MACE**

The median follow-up period was 6.3 years (interquartile range [IQR] 4.3–9.0). In total, 996 individuals experienced a MACE during the follow-up period. We found an association between higher HbA1c variability and incident MACE (Table 2) (HR 1.09 [95% CI 1.03–1.15]; \( P = 0.002 \), per 1-SD [0.09%] increase in HbA1c variability). Associations between interaction effect of index HbA1c and trend categories and hazard of MACE (P value for effect modification = 0.014) are summarized in Table 2. We calculated absolute 5-year risks of MACE for selected combinations of age-, sex-, and disease-specific risk factors (Fig. 1) along with extreme risk profiles (Supplementary Fig. 5) and found that the risk of MACE increased with increasing levels of HbA1c variability.

**HbA1c Variability and Risk of All-Cause Mortality**

A total of 856 individuals died during follow-up. We found an association between higher HbA1c variability and the hazard of all-cause mortality (HR 1.13 [95% CI 1.07–1.20]; \( P < 0.001 \), per 1-SD increase in HbA1c variability). We found that the effect of trend on the hazard of all-cause mortality also depended on
index HbA1c (P value for effect modification = 0.013). For example, the HR for a decreasing trend compared with a stable trend was 1.92 (95% CI 1.14–3.21; P = 0.013) (Table 2) in a low index HbA1c category, 1.54 (95% CI 1.12–2.13; P = 0.009) in a moderate index HbA1c category, and not significant (1.03 [95% CI 0.81–1.29]; P = 0.825) in a high index HbA1c category. We calculated absolute 5-year risks of all-cause mortality for selected combinations of age, sex, and disease-specific risk factors (Supplementary Fig. 6) along with extreme risk profiles (Supplementary Fig. 5) and found that HbA1c variability generally conferred a lower absolute risk of all-cause mortality compared with the risk of MACE, at least in people <70 years of age (Fig. 1 and Supplementary Fig. 6).

HbA1c Variability and Risk of Incident Type 2 Diabetes
During follow-up, 1,267 individuals developed type 2 diabetes. We observed no statistically significant association between HbA1c variability and type 2 diabetes (HR 1.00 [95% CI 0.95–1.05]; P = 0.935, per 1-SD increase in variability) (Table 2). We found significant interaction effect between index HbA1c and trends and type 2 diabetes (P value for effect modification <0.001), where increasing trend compared with a stable trend conferred an increased hazard of type 2 diabetes, whereas a decreasing trend was associated with a decreased hazard of type 2 diabetes, for each category of index HbA1c (Table 2).

Interaction Analyses
We found no statistically significant interactions between HbA1c variability and age-groups, sex, hypertension, CCI, index HbA1c and trend, for outcome MACE, all-cause mortality, and type 2 diabetes.

Conclusions
We are, to the best of our knowledge, the first to describe an association between glycemic variability and the risk of incident MACE and all-cause mortality in a population without diabetes.

We found that HbA1c variability, measured as the SD of the residuals, is significantly associated with increased risks of incident MACE and all-cause mortality, independent of traditional cardiovascular risk factors, index HbA1c, and trend. The same effects were observed for random plasma glucose variability. We did not observe an association between HbA1c variability and incident type 2 diabetes, but as expected, combinations of index HbA1c and HbA1c trend were significantly associated with incident type 2 diabetes. Our findings suggest that glycemic variability may contain valuable prognostic information for the outcomes of MACE and mortality, even

### Table 1—Baseline characteristics of the study cohort

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Total</th>
<th>Decreasing</th>
<th>Stable</th>
<th>Increasing</th>
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<tbody>
<tr>
<td>HbA1c variability, median (IQR)</td>
<td>0.08 (0.04, 0.15)</td>
<td>0.10 (0.04, 0.17)</td>
<td>0.07 (0.03, 0.13)</td>
<td>0.09 (0.04, 0.16)</td>
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<td>Age (years), median (IQR)</td>
<td>64.9 (56.3, 72.6)</td>
<td>64.7 (56.4, 72.6)</td>
<td>65.1 (56.4, 72.2)</td>
<td>64.7 (56.2, 72.2)</td>
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<td>Male, n (%)</td>
<td>2,864 (42.4)</td>
<td>636 (43.5)</td>
<td>1,455 (41.8)</td>
<td>773 (42.7)</td>
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<tr>
<td>Average annual income, n (%)</td>
<td>First tertile 2,252 (33.3)</td>
<td>444 (30.4)</td>
<td>1,173 (33.7)</td>
<td>635 (35.1)</td>
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<td>Hypertension, n (%)</td>
<td>3,216 (47.6)</td>
<td>668 (45.7)</td>
<td>1,676 (48.1)</td>
<td>872 (48.2)</td>
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<tr>
<td>Obesity, n (%)</td>
<td>241 (3.6)</td>
<td>50 (3.4)</td>
<td>114 (3.3)</td>
<td>77 (4.3)</td>
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<tr>
<td>Smoking, n (%)</td>
<td>417 (6.2)</td>
<td>88 (6.0)</td>
<td>234 (6.7)</td>
<td>95 (5.2)</td>
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<td>CCI, n (%)</td>
<td>5,117 (75.7)</td>
<td>1,088 (74.4)</td>
<td>2,635 (75.7)</td>
<td>1,394 (77.0)</td>
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Variability is defined as the SD of the residuals from the linear regression. Index HbA1c corresponds to the intercept and categorized in low (<5.3% [34 mmol/mol]), moderate (5.3–5.7% [34–39 mmol/mol]), and high (≥5.7% [39 mmol/mol]) index HbA1c. Trend corresponds to the slope estimate and is categorized in decreasing (less than –0.11%/year), stable (~0.11 to 0.11%/year), and increasing trend (>0.11%/year). Average annual income corresponds to mean annual gross income during a 5-year period prior to baseline.
in individuals with HbA1c levels within normal range.

Several pathophysiological mechanisms may be involved in the observed association between visit-to-visit glycemic variability and MACE and all-cause mortality. Mechanistic animal and human studies have mainly focused on short-term variability in blood glucose, and studies on long-term variability, as reflected in HbA1c levels, have not been conducted. However, intermittent high blood glucose exposure, rather than chronic hyperglycemia, has been shown to have deleterious effects on endothelial function, mediated through oxidative stress (14,31). In vitro studies have shown that intermittent high glucose levels stimulate overproduction of reactive oxygen species, which leads to increased cellular apoptosis in human umbilical vein endothelial cells compared with a constant high-glucose environment (14,15). Furthermore, in vivo studies on diabetic rats have shown that rats with induced “glycemic swings” have higher levels of oxidative stress markers and endothelial dysfunction, compared with rats with sustained hyperglycemia (16). Fluctuations in blood glucose concentrations have also been associated with an increase in circulating inflammatory cytokines, and accelerated macrophage adhesion to endothelial cells, stimulating the progression and formation of fibrotic atherosclerotic lesions (32,33).

The association between HbA1c variability and cardiovascular events that we report could reflect that HbA1c is a proxy for other systemic conditions that increase cardiovascular risk. Such systemic conditions could in theory lead to generalized frailty, in which higher variability in numerous biological parameters confer risk through parallel pathological pathways. We have tried to account for this through various sensitivity analyses. First, we performed a sensitivity analysis including only individuals without significant diseases. Hence, subjects with severe systemic conditions such as cancer and kidney and liver disorders were excluded at baseline. Second, we performed a sensitivity analysis where individuals who increased in CCI during the inclusion period were excluded. As such, we accounted for individuals with an a priori increased risk of cardiovascular events due to a more rapid decline in overall health status. Although our study design does not allow for casual inference, both of these sensitivity analyses gave results that were very similar to the main analyses (Fig. 2), supporting that the associations we report are not driven by yet undiagnosed severe systemic disorders.

We believe the present results may highlight an important issue. Patients with large fluctuations in HbA1c levels, although with absolute measurement values within normal range, may be falsely reassured of their low cardiovascular risk, based on their HbA1c residing within standard guideline limits. However, our results warrant replication in larger studies, and future long-term studies are needed to examine whether strategies to reduce variability in HbA1c can effectively reduce risk of cardiovascular disease in subjects with HbA1c within the normal range.

In this study, we used three consecutive annual measurements of HbA1c in the normoglycemic range, allowing for a more detailed estimation of the effect of longitudinal trajectories within HbA1c categories. Although not statistically significant for higher HbA1c levels, we found indications of a J-shaped relationship between trend in HbA1c and all-cause mortality, which is consistent with previous studies (34–36). We also found an

<table>
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<th>Table 2—Results of event-specific Cox regression</th>
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<td>Outcome</td>
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<td>MACE</td>
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<td>Low index HbA1c</td>
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<td>Increasing vs. stable trend</td>
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<td>All-cause mortality</td>
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<td>Type 2 diabetes</td>
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<td>Increasing vs. stable trend</td>
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Shown are effects of HbA1c variability, interaction effects of index HbA1c, and trend categories. HbA1c variability is reported in HR per SD increase in variability. Index HbA1c corresponds to the intercept and categorized in low (<5.3% [34 mmol/mol], moderate [5.3–5.7% [34–39 mmol/mol], and high (>5.7% [39 mmol/mol]) index HbA1c. Trend corresponds to the slope estimate and is categorized in decreasing (less than −0.11%/year), stable (−0.11 to 0.11%/year), and increasing trend (>0.11%/year). Interaction effects are assessed within each category of index HbA1c, comparing increasing/decreasing trend category with stable trend (reference) for each outcome. The Cox model was additionally adjusted for age-groups, sex, hypertension, and CCI.
increased risk of MACE in individuals with low HbA1c levels and a decreasing trend. Suggestive risk increase in the lower glycemic region has also been reported in previous studies of adults without diabetes (37–39). Our results suggest that clinicians should be attentive to individuals not only with increasing levels but also decreasing levels of HbA1c over time, in particular to those with initially low HbA1c levels.

Strengths of this study include a large sample size, data on multiple visit-to-visit measurements of HbA1c, and up to 13 years of follow-up. Despite this, this study has important limitations. First, the successive HbA1c values were collected from available laboratory records as a part of the patient’s routine clinical follow-up, and the indication for HbA1c referral is unknown. In Denmark, general practitioners refer patients for blood sampling based on a variety of clinical indications or as part of an annual health check. Rather than carefully selecting individual blood tests, patients may be referred for a collection of blood samples, in which HbA1c is included. As such, many primary care patients will have HbA1c measured on indications other than screening for diabetes. Second, we were unable to control for alternating diets or lifestyle interventions causing fluctuating weight, which could have influenced the variability observed in the study. However, we adjusted for the individual HbA1c trend over time to account for an overall decline or improvement in health or lifestyle interventions. Third, we did not have direct access to clinical variables important for MACE and death, such as BMI and smoking. However, we were able to account for this by using registry diagnoses for obesity and smoking, although we acknowledge that these conditions are underreported in the registries. Last, there is a potential for selection bias.

Figure 1—Five-year absolute risk prediction chart for MACE, based on individuals with a stable trend and moderate index HbA1c group, and the combination of risk factors, with respect to different levels of HbA1c levels (%). The color scheme refers to the absolute 5-year risk (%) for MACE. (A high-quality color representation of this figure is available in the online issue.)
due to exclusion of individuals without annually spaced measurements. This limitation is inherent to the study design, which diminishes the effect of time between successive values on the variability index. We found that included individuals were significantly older, had higher levels of HbA1c, and had more concomitant medications compared with excluded individuals (Supplementary Table 6). However, we found similar degrees of comorbidity between the included group and the excluded group (with three or more HbA1c outside design period). Since we used three (and four) annual HbA1c measurements, our results can only be extrapolated to other populations without diabetes with the same number of measurements.

In conclusion, we found an association between high HbA1c variability and increased risks of incident MACE and death from all causes. Both findings were independent of traditional cardiovascular risk factors, index HbA1c levels, and trends in HbA1c.

**Funding.** J.G. was supported by the Research Foundation at Copenhagen University Hospital, Rigshospitalet, Karen Marie Jespersen og Datters Legat, and the John og Birthe Meyer Foundation. J.B.N. was supported by grants from the Danish Heart Foundation (16-R107-A6779) and the Lundbeck Foundation (R220-2016-1434), Fonden til Lægevidenskabens Fremme (the A.P. Møller and Chastine Mc-Kinney Møller Foundation for General Purposes), and Fondsbørsveksleren Henry Hansen og Hustru Karla Hansen Født Vestergaards Legat.

**Duality of Interest.** L.K. has received speaker honorarium from Novartis. J.H.S. has received research grants from Medtronic, Biotronik, and Gilead, personal fees as speaker for Medtronic, Biotronik, and Boehringer Ingelheim, and personal fees as a member of an advisory committee for Medtronic. A.G.H. is an employee of Novo Nordisk A/S, Denmark. No other potential conflicts of interest relevant to this article were reported.

**Author Contributions.** J.G., M.W.S., and J.B.N. made primary contributions to study conception, data analysis, interpretation of results, and writing of the manuscript. J.K.K. and J.L.I. contributed to the interpretation of results and helped write the first draft of the manuscript. P.B. and T.A.G. contributed to the study conception, design, and statistical analyses, wrote the statistical analysis plan, and contributed to the interpretation of the results. B.L. collected the data and contributed to the interpretation of results. S.H., L.K., J.H.S., M.S.O., and A.G.H. contributed to the interpretation of results. All authors reviewed and revised the manuscript critically for important intellectual content and approved the final version. J.B.N. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
References


