Biological risk factors and sleep-related risk factors of type 2 diabetes mellitus

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2019

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Chapter 3

A prospective study on glucagon responses to oral glucose and mixed meal and 7-year change of fasting glucose.

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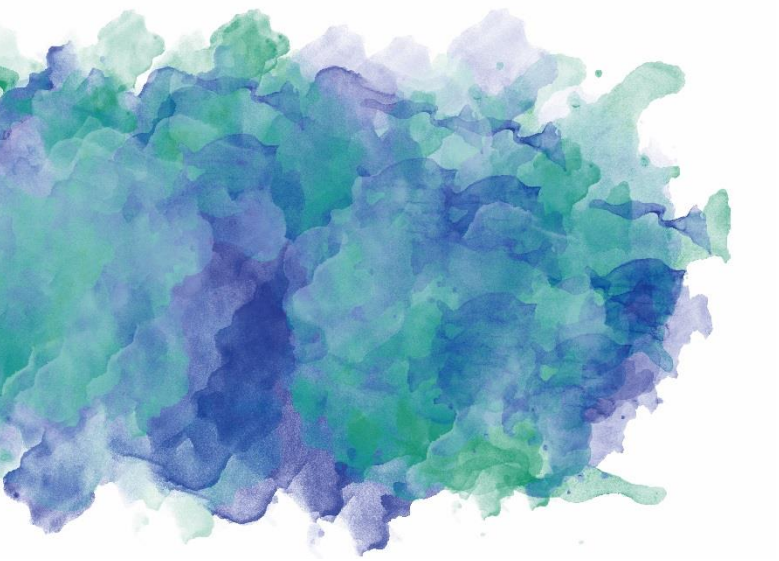
Clinical Endocrinology, 2019.



ABSTRACT

Introduction: The role of insufficient glucagon suppression after an oral load in the development of type 2 diabetes mellitus is unclear. The aim of this study was to examine the association between glucagon responses at baseline and fasting glucose levels 7 years later.

Methods: Data of the Hoorn Meal Study was used; an observational cohort study among 121 persons without diabetes with a mean age of 61.1 ± 6.7y and 50% being female. The glucagon response to an oral glucose tolerance test and mixed meal test was expressed as early and late incremental area under the curve. The association with change of fasting glucose levels at follow-up was assessed by linear regression analysis.

Results: The early glucagon response following the mixed meal test was associated with an increase in fasting glucose levels of 0.18 mmol/l (95%-CI: 0.04-0.31, p=0.01), per unit increase in the incremental area under the curve of glucagon, adjusted for confounders. No significant associations were observed for the late response after the mixed meal test or oral glucose tolerance test.

Conclusions: Within a population without diabetes, relative lack of glucagon suppression early after a meal was associated with increased glucose levels over time, suggesting a role of insufficient glucagon suppression in the deterioration of glycemic control.

INTRODUCTION

People with type 2 diabetes mellitus (T2DM) show higher fasting glucagon levels and reduced suppression of glucagon, compared to persons with normal glucose tolerance, which contribute to the hyperglycemia characterizing T2DM [1-5]. Several cross-sectional studies have shown that these abnormal glucagon responses already exist in people with prediabetes [1, 3, 6-12]. However, these cross-sectional designs are unable to determine if reduced suppression of glucagon precedes development of T2DM.

To date, only two small prospective studies have been conducted [13, 14], with contradicting results. Following an intravenous arginine challenge, a higher glucagon response in 86 participants with normal glucose tolerance was associated with higher 2-hour glucose concentrations three years later [13]. In contrast, in 50 participants part of a lifestyle intervention, less glucagon suppression after an oral glucose tolerance test (OGTT) was associated with improved insulin sensitivity after 9 months [14]. Given the conflicting results and small, restricted populations, more prospective studies in the general population are needed [15]. In addition, as next to carbohydrates, also fat and protein affect glucagon secretion [16], a mixed meal test (MMT) may be more relevant and a physiological challenge, compared to only an OGTT or arginine challenge, to assess glucagon response.

The growing interest in the use of glucagon-based therapies for diabetes [17] underscores the importance to determine the possible aetiological role of glucagon in T2DM development. Therefore, the present study investigates the glucagon responses to an OGTT and MMT at baseline in relation to change in fasting glucose levels 7 years later, in individuals who did not have diabetes at baseline. We hypothesize less glucagon suppression after an OGTT and MMT to be associated with increased glucose levels over time.

MATERIALS AND METHODS

Study population and study procedure

Study details on the participants are described in Koopman et al. 2008 [18]. In short, 208 men and women from the Hoorn Meal Study were included and 129 participants [18] were followed-up after 7 years. Compared to the participants who took part in the follow-up examination, those who declined participation had a significantly higher BMI and fasting glucose levels at baseline. For the current study, participants with T2DM at baseline or without glucose measures at follow-up were excluded. In addition, one participant with glucagon levels more than 3 standard deviations higher was excluded, resulting in a study population of 121 subjects. At baseline, participants

received a 5-point 75g-OGTT and a standardized 7-point MMT after an overnight fast, on separate days and provided in random order, within 2 weeks. The mixed meal consisted of two croissants, butter, Gouda cheese, full-fat milk and yogurt drink with soluble carbohydrates (maltose) and was completely consumed by all the participants. The nutrient content was 3487 kJ, 36 energy% carbohydrates, 52 energy% fat and 12 energy% proteins [1]. Details of the OGTT and MMT and other measures collected were described earlier [1, 18]. At follow-up, a physical examination was performed, including measurement of fasting glucose levels, lifestyle factors and anthropometry. All participants signed informed consent and the study was approved by the ethics committee 'METc VUmc'.

Dependent, independent and covariate variables

The dependent variable was fasting plasma glucose levels after 7y of follow-up. Fasting plasma glucose levels at baseline and follow-up were determined using the glucose hexokinase method Modular analytics, Roche diagnostics, Mannheim, Germany, with intra- and inter-assay coefficients of variation of 1.5% and 1.5%, respectively. The independent variable was glucagon responses to an OGTT and MMT. To determine glucagon levels, blood was collected at several time points during the OGTT and MMT, in EDTA tubes with aprotinin, which were stored on ice during processing. After processing, samples were stored at -20C, after which they were defrosted and analyzed using a radioimmunoassay (Linco Research, St Louis, MO, USA). The radioimmunoassay had the following characteristics: no cross-reactivity with insulin or prosinsulin, with inter- and intra-assay coefficients of variation of 8% and 14%, a recovery of 96-98% and lower limit of quantitation of 2.3 pmol/l. Glucagon responses following OGTT and MMT were expressed as the incremental area under the curve (iAUC) and calculated using the trapezoid method. The glucagon response was differentiated for early (0-30 min), late (30-120 min for OGTT and 30-240 min for MMT) and total response, which is in accordance with Færch et al. 2016[8], who showed that timing of glucagon suppression plays an important role in glucose regulation. Missing values for fasting levels of glucagon prior to OGTT (n=3) and MMT (n=1) and at 2h OGTT (n=3) and 4h MMT (n=3), fasting GIP (n=3) and GLP-1 (n=3) were substituted by median or mean imputation of that time point. Missing glucagon values at the other time points were substituted with the mean of the surrounding time points (OGTT; n=3, MMT; n=7) or, when not possible, substituted by median imputation.

Age, sex, follow-up duration, fasting glucagon and fasting glucose levels at baseline, BMI (at baseline and follow-up), fasting GIP and GLP-1 levels, OGIS and insulinogenic index were assessed as confounders. To assess insulin sensitivity, the validated oral glucose insulin sensitivity index (OGIS) was used [19]. The insulinogenic index was calculated by dividing the increment in insulin during the first 30 min by the

increment in glucose over the same period. Details of the measurement methods of the other confounders were described earlier [1, 18].

Statistical analyses

The association between glucagon responses at baseline and fasting glucose levels at follow-up was studied using linear regression analyses. We adjusted for age, sex, follow-up duration, fasting glucagon and fasting glucose levels at baseline (model 1) and BMI (at baseline and follow-up), fasting GIP and GLP-1, OGIS and insulinogenic index (model 2). Participants with normal glucose tolerance and impaired fasting glucose were studied together as we observed no significant effect modification of glucose metabolism status. Statistical analyses were performed with SPSS version 20.0 (SPSS Inc., Chicago, IL) and a p-value below 0.05 was considered to be statistically significant.

RESULTS

The characteristics of the study population are described in Table 1. At baseline participants had a mean age of 61.1 ± 6.7y and 50% were female. During a median of 7.0(0.2) years of follow-up, 18 participants progressed from normal glucose tolerance to impaired fasting glucose (14.9%) and 9 (7.4%) developed T2DM. In addition, an average increase of 0.43±0.5 mmol/l, 0.4±1.9 kg/m2 and -0.80±6.4 cm in glucose levels, BMI and waist circumference over the 7 year follow up period were observed, respectively. Figure 1 represents the absolute change in glucagon levels during the OGTT and MMT, which show a decrease of glucagon levels after the OGTT and increase after the MMT, respectively.

Associations with fasting glucose levels after 7 years of follow-up

The association between responses of glucagon at baseline and fasting glucose levels after 7 years are shown in Table 2. A higher early ($iAUC^{0-30}$) glucagon response after MMT was associated with a greater increase of 0.19 mmol/l (0.05;0.32) in fasting glucose levels at follow-up, adjusted for confounders. Additional adjustment for BMI, fasting incretin levels, OGIS and insulinogenic index (model 2) did not affect the results. For the early glucagon response ($iAUC^{0-30}$) after OGTT comparable results were observed, albeit not significant. Late and total glucagon responses after MMT and OGTT were not associated with fasting glucose at follow-up (Table 2).

Table 1. Population characteristics at baseline (n=121).

NGT/ IFG (n)	109/12
Sex (% female)	50
Age (years)	61.1 ± 6.7
Body mass index (kg/m^2)	26.7 ± 3.7
Fasting plasma glucose (mmol/l)	5.4 ± 0.4
2h plasma glucose OGTT (mmol/l)	5.0 ± 1.3
Fasting serum insulin (pmol/l)	40.9 (30.4)
Fasting plasma GLP-1(pmol/l)	10.8 (4.8)
Fasting plasma GIP (pmol/l)	6.3 (6.3)
Insulinogenic index OGTT	105.3 (101.4)
OGIS OGTT (ml/min per m^2)	415.4 ± 63.3
Fasting plasma glucagon (pmol/l)	9.40 (2.9)
Glucagon iAUC$^{0\text{-}30}$ OGTT (h·pmol/l)	-0.23 (0.7)
Glucagon iAUC$^{0\text{-}30}$ MMT (h·pmol/l)	0.48 (0.7)
Glucagon iAUC$^{30\text{-}120}$ OGTT (h·pmol/l)	-2.80 (3.8)
Glucagon iAUC$^{30\text{-}240}$ MMT (h·pmol/l)	2.13 (3.0)
Glucagon iAUC$^{0\text{-}120}$ OGTT (h·pmol/l)	-2.94 (4.5)
Glucagon iAUC$^{0\text{-}240}$ MMT (h·pmol/l)	2.51 (3.5)

Values are mean ± SD, or median (interquartile range) in case of a skewed distribution. NGT= normal glucose tolerance; IFG = impaired fasting glucose; ≥ 6.1 mmol/l; OGTT= oral glucose tolerance test; MMT= mixed meal test. GLP-1= glucagon-like peptide-1; GIP= glucose-dependent insulinotropic polypeptide; OGIS= oral glucose insulin sensitivity index; iAUC= incremental area under the curve. Hormone values are mean of OGTT and MMT or otherwise indicated. Negative values for iAUC indicate suppression.

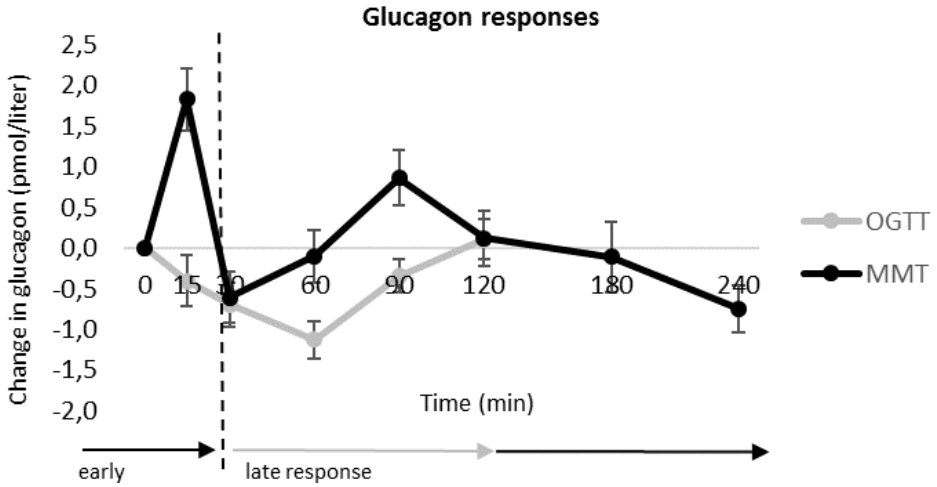


Figure 1. Absolute change (increase and decrease) in glucagon levels (mean with 95% CI) to OGTT (grey) and MMT (black) during baseline measurement in 121 subjects without diabetes.

Table 2. Linear regression coefficients (95% confidence interval) for the association of the iAUC of glucagon following OGTT and MMT at baseline and fasting glucose levels after 7.0 years of follow-up.

	Glucagon	Model 1	Model 2
Total response	**iAUC$^{0\text{-}120}$ OGTT**		
	B	-0.01 (-0.05 ; 0.04)	-0.01 (-0.05 ; 0.04)
	p-value	0.71	0.61
	iAUC$^{0\text{-}240}$ MMT		
	B	0.02 (-0.02 ; 0.05)	0.02 (-0.02 ; 0.05)
	p-value	0.28	0.28
Early response	**iAUC$^{0\text{-}30}$ OGTT**		
	B	0.13 (-0.05 ; 0.30)	0.10 (-0.08 ; 0.27)
	p-value	0.15	0.28
	iAUC$^{0\text{-}30}$ MMT		
	B	**0.19 (0.05 ; 0.32)**	**0.18 (0.04 ; 0.31)**
	p-value	**0.01**	**0.01**
Late response	**iAUC$^{30\text{-}120}$ OGTT**		
	B	-0.02 (-0.08 ; 0.03)	-0.02 (-0.07; 0.03)
	p-value	0.38	0.35
	iAUC$^{30\text{-}240}$ MMT		
	B	0.01 (-0.03 ; 0.05)	0.01 (-0.03 ; 0.05)
	p-value	0.64	0.59

B= regression coefficient; iAUC= incremental area under the curve; OGTT= oral glucose tolerance test; MMT= mixed meal test. **Bold** = significant association

Models:
1: Adjusted for fasting glucagon and fasting glucose levels at baseline, age, sex and follow-up duration
2: Adjusted for fasting glucagon and fasting glucose levels at baseline, age, sex, follow-up duration, BMI at baseline and at follow-up, fasting GIP and GLP-1 levels, OGIS and insulinogenic index

DISCUSSION

We showed that high early glucagon response (0-30 min) after MMT was associated with increased fasting glucose levels 7y later. Comparable results were observed for the early glucagon response after OGTT, although less pronounced and not significant. This is in line with most earlier cross-sectional studies [1, 3, 6-12], but not all [14], which observed a relative lack of glucagon suppression in people in prediabetes, suggesting that abnormal glucagon responses precede glycemic deterioration.

We only observed an association for the early response and not the late or total glucagon responses. This is in line with data from Faerch et al. 2016 [8] who also observed a relative lack of early glucagon suppression only during OGTT in participants with prediabetes. Comparison with the 2 earlier prospective studies [13, 14] is however hampered by study differences, with only women [13] or high risk populations [14] studied and islet function being examined using an arginine challenge [13]. The fact that the study of Wagner et al. 2017 [14] was embedded in a lifestyle intervention, which could have affected BMI and therefore the glucagon response and the fact there was only 9 months of follow-up, may also explain the difference with our findings and those by Larsson et al. 2000 [13].

As depicted in figure 1, which represents the absolute change in glucagon levels from baseline and supported by data from others [3, 11, 20-22], in general the glucagon response first increases after a mixed meal before being suppressed, while glucagon immediately decreases after an oral glucose load [4, 8-10]. This suggests a different sensitivity of the glucagon producing cells to various stimuli, with the main difference between OGTT and MMT being lipids and amino acids in the MMT, of which the latter is known to increase glucagon secretion [23]. This difference in sensitivity might be caused by interactions of glucagon with the other gut hormones such as GLP-1 and GLP-2. In turn, this sensitivity difference might also explain the difference in results on the association of the early glucagon response and fasting glucose at follow-up between OGTT and MMT.

Underlying mechanisms suggested for the hyperglucagonemia in T2DM are defective glucagon suppression by glucose and insulin, and insulin resistance in the alpha-cells [24]. In addition, incretin hormones, with a special focus on GLP-1, are thought to cause insufficient glucagon suppression [2, 24]. This hypothesis is supported by data from our group, which used the same cohort as the current study and showed a reduced GLP-1 response preceding glucose deterioration [18]. In the present study however, adjusting for fasting incretin levels, only marginally changed the association between glucagon response and fasting glucose at follow-up. Further research is therefore needed to elucidate the pathophysiology of the association between glucagon response and glucose over time.

Our study has some limitations, first, the number of participants examined is rather limited, albeit the largest study thus far. Because of this, and because of imputation of missing values, studies with larger sample sizes are required to confirm our results and to assess T2DM incidence. Second, our analyses are limited to (change in) fasting glucose levels at follow-up, not reflecting total glucose metabolism or insulin sensitivity status. Third, we measured glucagon using radioimmunoassay, while more recent studies used ELISAs. However, Albrechtsen et al. 2014 [25] showed that radioimmunoassay and ELISA measured similar glucagon levels in healthy or prediabetes individuals, with the assay only becoming dissimilar in individuals with abnormally high glucagon levels, which were not present in our sample. Fourth, as with any observational study, the baseline measurement of the exposure, the glucagon response, may not reflect the exposure during the (entire) follow-up period, and may have changed due to medication or diet. Unfortunately, no information on changes of diet or use of medication during follow-up was available. However, our results are adjusted for changes in BMI. Finally, the participants included in the analysis were slightly healthier compared to the participants who declined participation at follow-up and this could have led to an underestimation of observed associations.

One of the major strengths of this study is the prospective design with relatively long follow-up. Other strengths are the frequently sampled glucagon responses to OGTT and MMT which enabled us to distinguish the early and late response, for the first time after MMT as well. Finally, the extensive adjustment for possible confounding or mediating variables, including indicators of insulin sensitivity, beta-cell function and overweight, reduced the risk of potential confounding. These results provides the first evidence that targeting the early glucagon response might prevent T2DM development, suggesting a role for glucagon-based therapies in the prevention for diabetes [17]. However, additional studies are necessary.

In conclusion, within a population without diabetes, relative lack of glucagon suppression early after a meal was associated with increased glucose levels over time, suggesting a role of insufficient glucagon suppression in the deterioration of glycemic control.

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